PROTEOMICS OF CEREBROSPINAL FLUID IN PATIENTS DIAGNOSED WITH PNEUMOCOCCAL MENINGITIS

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BY

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DECLARATION

This thesis is the result of my own work. The material contained in the thesis has not been presented, nor is currently being presented, either wholly or in part for any other degree or other qualification

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Abstract

Streptococcus pneumoniae is the most common bacterial cause of community-acquired meningitis world-wide. Despite optimal antibiotic therapy and supportive care, the mortality of this condition remains very high at over 60% in under-resourced hospitals. There is an urgent need for more information about the mechanisms of brain damage and death in pneumococcal meningitis so that new treatments can be designed.

Using proteomic techniques and bioinformatics the protein content of cerebrospinal fluid was examined in great detail. A significant difference in the level of protein in normal CSF and clinically diagnosed meningitis was observed. 34 proteins were found to have an association with survival. Mass spectrometry allowed identification of these proteins. A subset of these identified proteins was validated using Western blot. In this thesis it has been observed that transferrin shows increased levels in CSF from non-survivors and complement C3 appears to show reduced levels in CSF from non-survivors in a larger sample size.

Animal models have added greatly to our knowledge of possible mechanisms of cell death and shown that hippocampal apoptosis and cortical necrosis are distinct mechanisms of neuronal death. The contribution of these pathways to human disease is unknown. Specific targets were selected from neuronal death pathways and from known pneumococcal proteins for confirmation in CSF using Western blot. In this study I will show that proteins associated with apoptosis are absent from CSF and there is low expression levels of proteins indicative of necrosis. In addition pneumococcal proteins remain at high concentrations in CSF after treatment. This information could lead to the design of novel therapies to minimise brain damage and lower mortality.

Publications

1. *Goonetilleke, U.R., Ward, S.A. and Gordon, S.B. (2009). Could Proteomic Research Deliver the Next Generation of Treatments for Pneumococcal Meningitis?* ***Interdiscip Perspect Infect Dis.***

2. *Goonetilleke, U.R., Scarborough, M, Ward, S.A. and Gordon, S.B. (2010) Proteomic Analysis of Cerebrospinal Fluid in Pneumococcal Meningitis Reveals Potential Biomarkers Associated with Survival.* ***J Infect Dis.***

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List of Abbreviations

2AAA.....Serine/Threonine phosphatase 2-alpha 65K regulatory chain
2D PAGE.....2 Dimensional Polyacrylamide Gel Electrophoresis
A1AT.....Alpha 1 antitrypsin precursor
ACN.....Acetonitrile
ACTH.....Adrenocorticotropic hormone
AIF.....Apoptosis Inducing Factor
ALBU.....Human Serum Albumin
ANR42.....Ankyrin protein 42
AP.....Alkaline Phosphatase
Apaf-1.....Apoptotic protease activating factor -1
APOH.....Beta-2-glycoprotein 1 precursor
ATP.....Adenosine Triphosphate
BBB.....Blood Brain Barrier
BCIP/NBT....5-bromo-4-chloro-3-indolyl phosphate/ nitro blue tetrazolium
BEGIN.....Brain-enriched guanylate kinase-associated protein
BSA.....Bovine Serum Albumin
C3/CO3.....Complement C3
Ca^{2+}.....Calcium ion
CAGE1.....Cancer associated gene 1
CBB.....Coomassie Brilliant Blue
CbpA.....Choline binding protein A
CD4.....Cluster of differentiation 4
CHAPS.....3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHIT1.....Chitotriosidase
CIA30.....NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9
CIQT9.....Complement C1q tumor necrosis factor-related protein 9
CKBB.....Creatine kinase Brain isoform
CNS.....Central Nervous System
CO_2.....Carbon Dioxide
C3/CO3.....Complement C3
CRP.....C-reactive protein
CSF.....Cerebrospinal fluid
CSTFT.....Cleavage stimulation factor 64 kDa subunit, tau variant
Da.....Dalton
DAB.....Diaminobenzadine tetrachloride
DESM.....Desmin
DNA.....Deoxyribonucleic acid
DTT.....Dithiothreitol
ECL.....Enhanced Chemiluminescence
EIF2S2.....Eukaryotic translation initiation factor 2, subunit 2 beta
ESI.....Electrospray ionisation
EST.....Expressed sequence tag

FADD.....Fas-associated death domain protein
FIBB.....Fibrinogen
FSCN1.....Fascin
GCS.....Glasgow coma scale
GDC.....Solute carrier family 25 (member 16)
GLOD4.....Glyoxalase domain-containing protein 4
GO.....Gene ontology
H_2O_2.....Hydrogen peroxide
HCl.....Hydrochloric acid
HIV.....Human immunodeficiency virus
HNRPC.....Heterogeneous nuclear ribonucleoproteins C1/C2
HPLC.....High Performance Liquid Chromatography
HPT.....Haptoglobin
HRP.....Horseradish peroxidase
IgG.....Immunoglobulin G
IPG.....Immobilised pH gradient
KE.....Kinetic energy
KPYM.....Pyruvate kinase
LC.....Liquid chromatography
LTA.....Lipoteichioic acid
LytA.....N-acetylmuramoyl-L-alanine-amidase
m/z.....Mass-to-charge ratio
mA.....milliampere
MALDI-TOF.....Matrix Assisted Laser/Desorption Ionisation Time of Flight
MMP.....Matrix metalloproteinase
Mr.....Relative molecular weight
MS.....Mass spectrometry
MS/MS.....Tandem mass spectrometry
Mw.....Molecular weight
NanA.....Neuraminidase A
NanB.....Neuraminidase B
NaOH.....Sodium hydroxide
NCP.....Nitrocellulose paper
nESI.....Nanoelectrospray
NL.....Non linear
NLF1.....Nuclear localized factor 1
O.D.....Optical Density
PAGE.....Polyacrylamide gel electrophoresis
PBP.....Penicillin Binding Protein
PCR.....Polymerase chain reaction
PGM5.....Phosphoglucomutase-like protein 5
pH.....Potentiometric hydrogen ion concentration
pI.....Isoelectric point
Pia.....Pneumococcal iron association
Piu.....Pneumococcal iron uptake

Ply.....Pneumolysin
PMF.....Peptide mass fingerprint
PMNL.....Polymorphonuclear leukocytes
PPAL.....Lysosomal Acid Phosphatase
PRS7.....26S protease regulatory subunit 7
PsaA.....Pneumococcal surface adhesin A
PSD.....Post source decay
PspA.....Pneumococcal surface protein A
PspC.....Pneumococcal surface protein C
PTM.....Post-translational modification
PVDF.....Polyvinylidene fluoride
RAB37.....Ras-related protein Rab-37
RF.....Radiofrequency
RN112.....Zinc finger protein 179
ROS.....Reactive oxygen species
RPM.....Revolutions per min
RXRG.....RXR gamma
SD.....Standard Deviation
SDS.....Sodium Dodecyl sulphate
STYL1.....Serine/threonine/tyrosine-interacting-like protein 1
TCPZ.....T-complex protein 1 subunit zeta
TH-Y.....Todd Hewitt Media supplemented with Yeast
TLR.....Toll like receptor
TNF-α.....Tumour necrosis factor alpha
TRFE.....Transferrin
UV.....Ultraviolet
v/v.....Volume per 100 ml solution
Vis.....Visible spectrum
w/v.....Weight per 100 ml solution
WHO.....World Health Organisation
YH004.....Tryptophan/serine protease
ZA2G.....Zinc alpha 2-glycoprotein precursor
ZIK1.....Zinc finger protein 1

CHAPTER 1

GENERAL INTRODUCTION

1.1 The Clinical Problem of Meningitis

Streptococcus pneumoniae is the most common pathogen associated with bacterial meningitis beyond the neonatal period (1). Pneumococcal meningitis has a high fatality rate of 19 – 37% even with optimal treatment and is much worse in under resourced nations (2). For example pneumococcal meningitis in Malawi has a high fatality rate of 61% (3). Survivors of the infection often develop long-term neurological sequelae, including hearing loss and other focal neurological deficits (4).

Pneumococci are able to colonise the nasopharynx without developing any serious consequences. Pneumococcal carriage rates in young children vary from over 40% in developed nations such as USA. to 87% in developing nations such as Gambia (5;6). Pneumococcal carriage rates in adults vary from approximately 10% in developed nations such as USA. to 51% in developing nations such as Gambia (7). When pneumococci spread to the sinuses, ear, lung and blood stream, diseases such as sinusitis, Otitis media, pneumonia and septicaemia can result (Figure 1.1).

1.2 The Pneumococcus

1.2.1 History of the Pneumococcus

Streptococcus pneumoniae is an ovoid gram-positive bacterium which was identified more than one hundred years ago as the causative agent of pneumonia (8). It is responsible for millions of deaths worldwide, in particular, across the African 'meningitis belt' (an area which stretches from Ethiopia through to Senegal). This hyper-endemic area is characterised by particular climate and social habits.

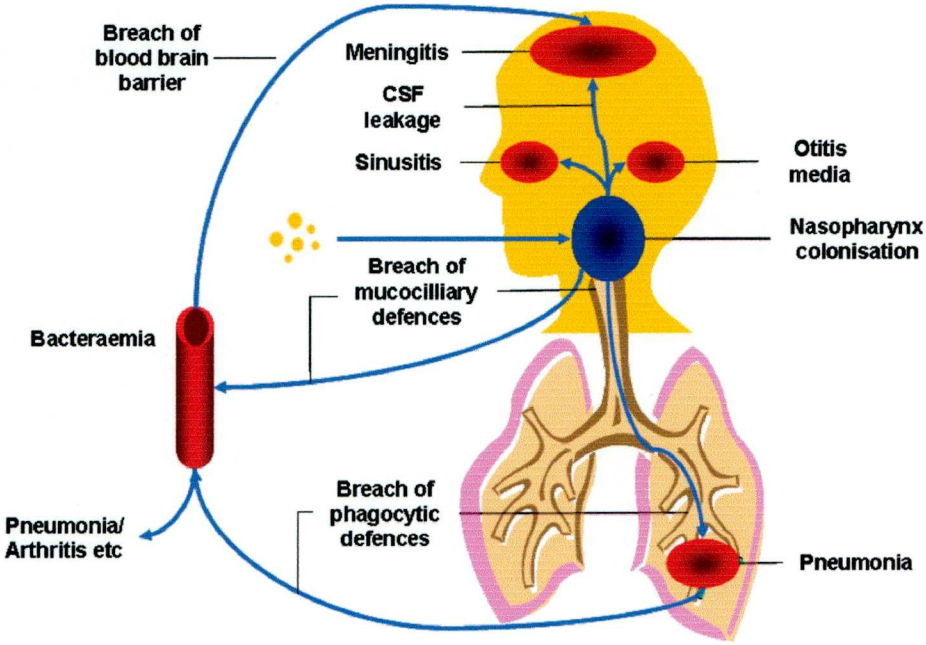

Figure 1.1 Outcome of pneumococcal invasion. *When pneumococci spread to the sinuses, ear, lung and blood stream, diseases such as sinusitis, Otitis media, pneumonia and septicaemia can result. Invasion of the central nervous system (CNS) by colonising pneumococci follows an alteration in the balance between the virulence of the bacteria and the defences of the patient. Factors such as common colds or other upper respiratory virus infections alter the lining of the respiratory tract and allow bacteria to enter the bloodstream. Pneumococci then actively translocate across intact endothelial layers by means of specific receptor binding and translocation. Endothelial cells normally separate the blood from neuronal tissue forming a protective blood-brain barrier (BBB). The integrity of the BBB is compromised by apoptosis of endothelial cells. The BBB breakdown allows further invasion of cerebrospinal fluid (CSF) (9).*

1.2.2 Pneumococcal Serotypes

Pneumococci have a highly developed capsule of great variation in chemical composition and of type specific capsular polysaccharides. Consequently pneumococci have at least 91 serologically distinguishable capsular polysaccharide types which comprise 48 cross-reactive groups (10).

The serotyping scheme for pneumococci has been updated many times, and the 91 currently recognized serotypes probably represent a high proportion of the total capsule diversity in the species, although a variant within serogroup 6 (serotype 6C) has been reported (11).

Among the 91 different pneumococcal serotypes, only a few account for the majority of invasive pneumococcal diseases and their relative frequency depends on age and sex and varies geographically (12). Regional differences in the prevalence of specific serotypes highlight the importance of knowing the serotypes for each geographic area (13).

These capsules are hydrated, negatively charged shells (except 7F, 7A, 14, 33F, and 37 species) which control diffusion, adherence and formation of biofilms and microcolonies.

1.3 Pathogenesis of Pneumococcal Meningitis

1.3.1 Signs and Symptoms of Pneumococcal Meningitis

As with other forms of bacterial meningitis, pneumococcal meningitis is characterised by headache, fever, nuchal rigidity and altered mental status. The petechial rash associated with meningococcal meningitis, however, is absent in pneumococcal meningitis. Few patients have all the symptoms but almost all patients (95%) have at least two of the four symptoms (4).

1.3.2 Pneumococcal Invasion

Pneumococci colonise the nasopharynx and attach to the nasopharyngeal cells of the upper and lower respiratory tract (14). This is facilitated by the presence of choline binding protein A (CbpA or pneumococcal surface protein C, PspC) on the cell wall of the pneumococcus (15). Invasion by colonising pneumococci follows an alteration in the balance between the virulence of the bacteria and the defences of the patient (16). Factors such as common colds or other upper respiratory virus infections alter the lining of the respiratory tract and aid pneumococcal translocation across intact epithelial layers (17). This is achieved by means of specific receptors such as the polymeric immunoglobulin receptor and platelet activating factor receptor (16;18;19).

1.3.3 Translocation of Pneumococci into the CNS

Endothelial cells normally separate blood from neuronal tissue forming a protective blood-brain barrier (BBB). The integrity of the BBB is compromised by apoptosis of endothelial cells. The BBB breakdown allows further invasion of cerebrospinal fluid (CSF) (20-22). It has been observed in some children, that bacteria can translocate directly from the nasopharynx into the CNS via olfactory neurones (23). A non-haematogenous route has also been demonstrated in animal models (24).

1.3.4 Outcome of CSF Host Inflammatory Response

The initial host inflammatory response is initiated by pneumococcal capsular polysaccharides and surface proteins, such as pneumolysin and PspA (25;26). Most of the tissue damage associated with meningitis is caused by host response. This may include the action of phagocytes, secreted granular toxins, cytokines, leukotrienes, matrix metalloproteinases and the direct pressure effect of cerebral oedema causing ischaemia (27).

1.3.5 Neuronal Cell Death and Meningitis

Pneumococcal proteins have been shown to contribute to neuronal cell death in animal models (28). For example pneumolysin can induce apoptosis of microglial and hippocampal neurones in a rat model at concentrations as low as 0.1 μgml^{-1} (29). Neuronal cell death has been determined to occur via three distinct pathways (30);

(i) Classic caspase-3–dependent cell death which leads to apoptosis or programmed cell death.

(ii) Caspase-3-independent cell death which leads to pyknosis (irreversible condensation of chromatin in the nucleus of a cell undergoing programmed cell death or apoptosis).

(iii) Necrosis, the unnatural death of cells and living tissue through cell swelling, chromatin digestion, and disruption of the plasma membrane and organelles.

Neuronal Cell Death in the Hippocampus

Animal models have been used to determine the mechanism of pneumococcal related neuronal apoptosis. In the rabbit model of pneumococcal meningitis hippocampal apoptosis was found to be the predominant form of neuronal damage (31;32). Inhibition of phosphorylcholine synthesis in mitochondria of neurons in the hippocampal dentate gyrus leads to mitochondrial release of apoptosis inducing factor (AIF) which in turn causes pyknosis of the hippocampus. In an adult mouse model both caspase-dependent and independent forms of neuronal cell death have been described in the dentate gyrus of adult mice (33). Bacterial cell wall products initiate mitochondrial release of cytochrome c leading to classic toll-like receptor (TLR) dependent-caspase-3 mediated apoptosis occurring more widely in the brain. Infant rat models of pneumococcal meningitis showed similar neuronal damage patterns to human disease (34;35). Also in the infant rat meningitis model

apoptosis and pyknosis of neurons have been identified in the dentate gyrus of the hippocampus. In humans evidence of apoptosis has been observed in the dentate gyrus. The location of the dentate gyrus in the human brain is shown in Figure 1.2 (36). Apoptosis primarily affects the subgranular zone containing recently divided immature neurons. Pyknosis occurs throughout the dentate granular cell layer. Both mature and immature neurons are affected as a result (34).

Neuronal Cell Death in the Cortex by Necrosis

A feature of severe pneumococcal meningitis is ischemic damage of neurons in the ischemic core of the cortex which results in necrosis in addition to caspase-3 dependent cell death (34;37;38). Inflammation of the meninges leads to oxygen and glucose deprivation of neuronal cells. The release of excitatory neurotransmitters from glutamatergic neurons lead to glutamate receptor overactivation. This leads to Ca^{2+} influx, subsequent injury and eventually neuronal necrosis (39). Energy depletion is a potent trigger of necrosis. During ischemic and hypoglycaemic episodes, lack of oxygen or essential nutrients leads to a shortage of energy to sustain the membrane potential (40). Necrosis has been commonly thought of as a passive process but neuronal necrosis and neuronal apoptosis may share a final common path i.e. the mitochondrial pathway as illustrated in Figure 1.3 (41;42).

Creatine kinase brain type (CKBB) may act as a marker of neuronal cell death by necrosis. The small size of the dimeric enzyme means it can leak out of cells under ischemic episodes or injury to the brain. CKBB in the CSF originates from neurons and astrocytes (43). CKBB activity in CSF has been shown to be elevated in neurological diseases including infectious conditions (44).

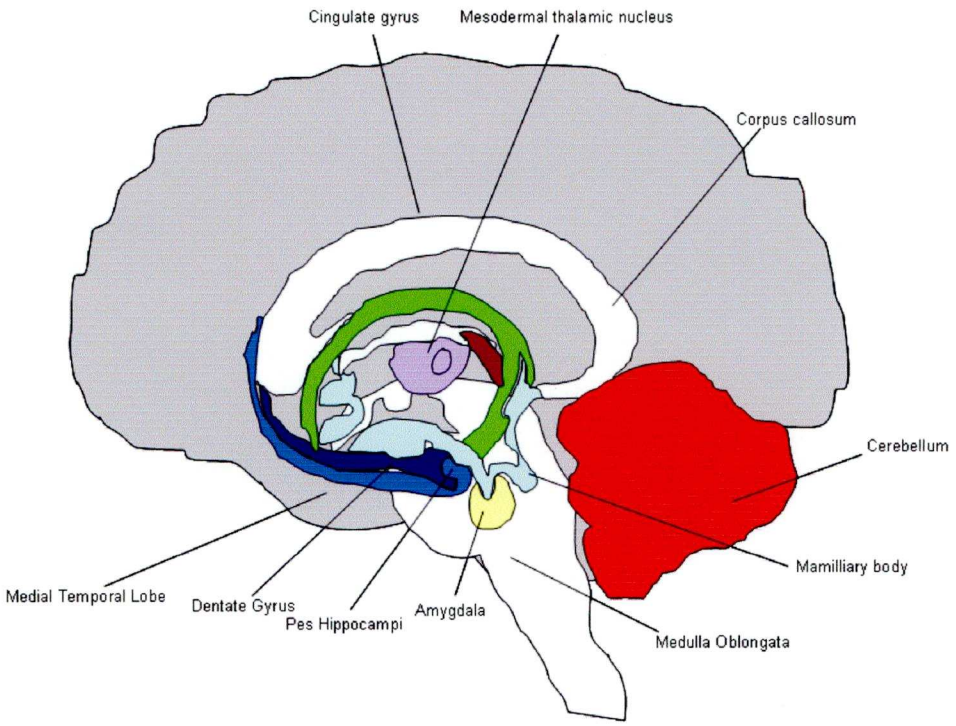

Figure 1.2 Potential cell death regions of the human brain. *The hippocampus is a part of the forebrain, located in the medial temporal lobe. The hippocampus is used in storing and processing spatial information. In the rabbit model of pneumococcal meningitis, hippocampal apoptosis was found to be the predominant form of neuronal damage. In humans apoptosis and pyknosis have been identified in the dentate gyrus of the hippocampus. Apoptosis primarily affects the subgranular zone containing recently divided immature neurons. Pyknosis occurs throughout the dentate granular cell layer (9)*

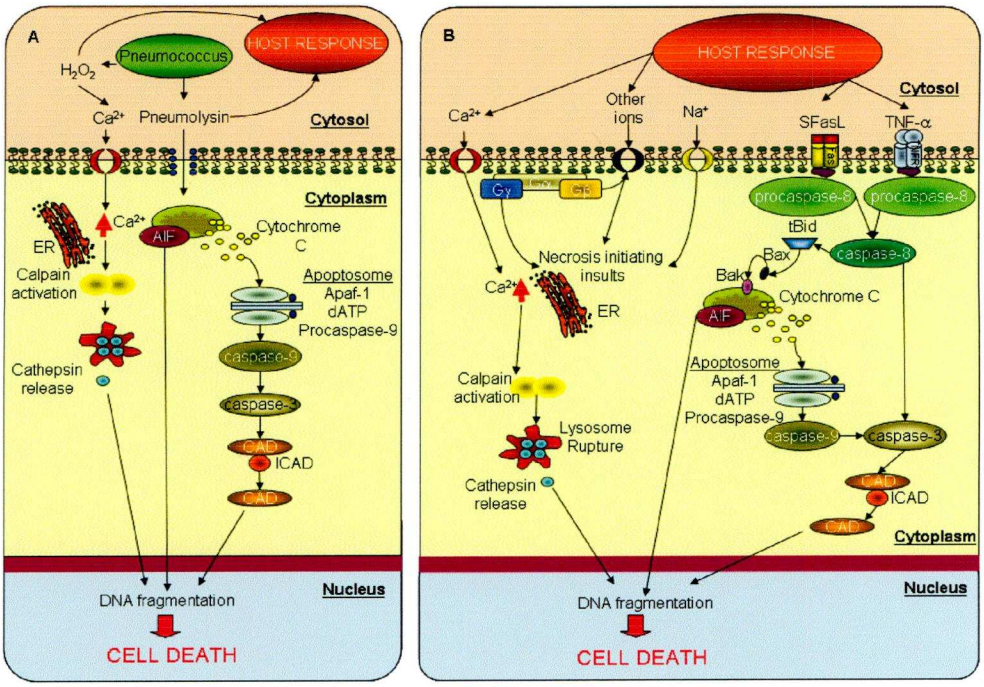

Figure 1.3 Pathways leading to cell death. ***A*** *The cell wall of S. pneumoniae has a diverse protein population. Proteins such as pneumolysin at a concentration of 0.1μgml^{-1} can trigger apoptosis on entering cells by targeting mitochondria. In addition oxidising components such as hydrogen peroxide can trigger apoptosis and necrosis.* ***B*** *The adaptor protein FADD activates caspase-8 to form a signal complex to directly activate caspase-3. The mitochondrial stress pathway is initiated when proapoptotic proteins in the cytoplasm stimulate the rupture of the mitochondria. In the caspase dependant pathway, cytochrome c released from the mitochondria forms a complex in the cytoplasm with ATP and apoptotic protease activating factor -1 (Apaf-1) and activated caspase-9 called an apoptosome which activates caspase-3. The caspase independent pathway (pyknosis) is as a result of apoptosis inducing factor (AIF). The necrotic pathway is activated in severe meningitis. Alterations in the concentration of cytoplasmic calcium could signal the mobilisation of executioner cathepsin proteases and other hydrolases, through calpain activation (9).*

1.3.6 Host and Pathogen Proteins

Proteomic comparisons of infected and normal CSF can be expected to differ in the concentration of both pneumococcal proteins and host proteins. Identification of pneumococcal proteins associated with poor outcome may suggest either therapeutic possibilities or vaccine candidates. Host proteins associated with poor outcome may suggest pathways amenable to immunomodulation or therapeutic intervention.

Pathogen Proteins

Proteins associated with pneumococci may be found in the pneumococcal capsule or the cell wall.

Capsule

Pneumococci have developed a unique survival mechanism in order to evade the immune system. The capsule functions to protect the pneumococcus from phagocytosis by polymorphonuclear leukocytes, by physically shielding the inner structures from antibodies, separating bound antibodies and complement receptors on phagocytes (45;46).

Pneumococci which lack the capsule are normally avirulent and antibodies to the capsular polysaccharides are protective (47). The capsule as such does not cause inflammation (48). Oxygen levels were discovered to affect the opaque (O) and transparent (T) phase variants differently (17). Greatly enhanced production of capsular polysaccharide in O variants in conditions of reduced oxygen such as the blood circulatory system where discovered, whereas synthesis of capsular polysaccharide in T variants remain comparatively low, under both aerobic and anaerobic conditions such as the nasopharynx (17).

Cell Wall and Cell Wall Polysaccharide

Pneumococci produce the antigenic polysaccharides teichioic acid and lipoteichioic acid (Forssman antigen). These structures are unique because they possess identical repeat and chain structures, unlike other gram positive bacteria, in which they are different in both structure and synthesis (49). Choline present within the structural cell wall plays a key role in many of the functions of the organism. Replacement of the choline residues with the structural analog ethanolamine causes dramatic changes in the cell physiology such as growth in long chains, loss of competence for transformation, resistance to lysis etc. The exposed phosphocholine acts as an adhesion ligand for pneumocytes and peripheral endothelial cells. This also involves the platelet-activating-factor receptor which enhances adherence and penetration of host cells (49).

Pneumococcal Proteins

The cell wall of *S. pneumoniae* has a diverse protein population, and pathogenic expression of pneumoccal proteins are associated with adherence to and colonisation of mucosal surfaces, resistance to specific and non-specific host defences, penetration and invasion of host tissues, and generation of tissue damage mediated either directly by toxins or indirectly via inflammatory responses as summarised in Table 1.1. All the proteins listed in Table 1.1 have been described in experimental studies including animal models of meningitis and have been found to exhibit an effect on inflammation and toxicity e.g. *N*-acetylmuramoyl-L-alanine-amidase (LytA) is an autolytic enzyme required during cell division. Its role in pneumococcal meningitis is unknown but has been shown in various animal models to mediate toxicity and inflammation (50).

Proteins such as pneumolysin can stimulate the host response and also enter cells though pore formation. It has the ability to trigger apoptosis on entering cells by destruction of the mitochondria (50). In

Table 1.1 Pneumococcal proteins associated with infection

Protein	*Description*	*Action*	*References*
LytA	Enzyme required during cell division	Hydrolyses amide bonds between muramic acid and L-Alanine residues	(50;51)
PspA	Anchored to the outer layer of the plasma membrane	Inhibits complement activation	(52)
pneumococcal histidine triad (Pht)	Novel family of cell surface-exposed proteins	Induces antibodies capable of protecting mice against pneumococcal sepsis and death	(53;54)
PspC	Surface protein	Mediates invasion across human nasopharyngeal epithelial cells	(15;55)
Neuraminidases e.g. NanA and NanB	Cleaves terminal sialic acid residues	Unknown	(56;57)
Heat shock proteins	A highly conserved set of proteins	Produced as a result of temperature shift	(58)
hyaluronate lyase (Hyal)	Covalently linked to the cross-bridges of the cell wall peptidoglycan	Degrades essential components of the host's extracellular matrix (ECM)	(59)
pneumococcal surface antigen A (PsaA)	34.5 kDa protein covalently anchored to the cell membrane	Belongs to an ATP binding cassette (ABC)-type transport system	(60)
pneumolysin (Hemolysin or Ply)	53-kDa protein	Induces leakage of solutes after insertion into the lipid bilayer leading to membrane destablisation altering the membrane potential of the cell	(61)
penicillin-binding proteins (PBPs)	*S. pneumoniae* carry a relatively simple set of six PBPs	Catalyse the polymerisation of glycan chains and transpeptidation of pentapeptidic moieties	(62)
pneumococcal iron uptake (Piu) and iron acquisition (Pia)	Lipoprotein components of iron ABC transport systems	Essential for iron uptake	(63)

The Pneumococcal proteins above have been identified as being important contributors to the progression of pneumococcal disease and in particular in animal models of meningitis.

addition oxidising components such as hydrogen peroxide can also trigger apoptosis and necrosis. Both pneumolysin and autolysin have been shown to play a crucial role in the pathogenesis of pneumococcal meningitis in an adult rat model of meningitis (64).

Neuraminidases are a group of enzymes which can cleave terminal sialic acid residues from a wide variety of glycan structures (56). The pneumococcus produces two distinct neuraminidases, *N*-acetylneuraminic acid (NanA) and *endo*-β-1, 4-*N*-acetylglucosaminidase (NanB) (65;66). There are several conflicting publications on the precise role of NanA in pneumococcal disease, however an Otitis media chinchilla model revealed NanA-deficient pneumococci are significantly less able to colonise and persist in the nasopharynx and middle ear than NanA-sufficient wild-type pneumococci (57). The relative contribution of NanB to disease has not been reported in either a sepsis or pneumonia model.

Host Defence Proteins

The host immune response will most likely make up the majority of proteins present in the CSF because these proteins will include host immune response factors such as complement and cytokines, as well as specific immunoglobulins and proteins from serum leaking to the CSF as a result of the blood brain barrier breakdown (Table 1.2). Host proteins that this thesis will aim to find include:

Complement Cascade Proteins

In the absence of specific antibodies, activation of the complement cascade is imperative for the clearance of pneumococci. Many pathogenic serotypes are able to resist phagocytosis by being poor activators of the alternative complement pathway and by degrading the deposited C3b into opsonically less active components (67;68). The alternative pathway may

Table 1.2 Selected Proteins Associated with the Host Immune Response

Protein	*Description*	*Action*	*References*
Complement components e.g. C3b, iC3b, or C4b (CR1, CR3).	Consists of a number of small proteins found in the blood, normally circulating as inactive zymogens	Help clear pathogens from an organism.	(46)
IL-6	A pro-inflammatory cytokine.	Secreted by T cells and macrophages to stimulate immune response to trauma, leading to inflammation.	(69)
Interleukin-1 (IL-1)	A superfamily consisiting of IL-1α, IL-1β, and the IL-1 Receptor antagonist (IL-1RA)	They control lymphocytes. IL-1α and IL-1β are produced by macrophages, monocytes and dendritic cells	(70)
IgG	The most abundant immunoglobulin. Equally distributed in blood and in tissue liquids.	Activates complement (classic pathway), opsonization for phagocytosis and neutralisation of their toxins.	(71)

Host immune response factors such as complement and cytokines, as well as specific immunoglobulins and proteins from serum leaking to the CSF have all been described as having an association with survival in meningitis. The table above shows 4 proteins selected from the immune response which have been identified in analyses of the CSF samples used in this study.

be triggered by the capsular polysaccharides of some serotypes and by capsular polysaccharides.

C-reactive protein (CRP) is an acute phase protein which may interact with phosporylcholine residues of the capsule polysaccharide and activate the classical pathway of complement. In exponentially growing bacteria, the potential deposition of C3b to the cell wall excludes it from interacting with phagocytes since capsular polysaccharide is concealed by the capsule. Therefore phagocytosis of serotypes which lack phosphorylcholine in their capsule is not enhanced by a capsular polysaccharide or CRP mediated complement cascade. Transparent and opaque phenotypes may differ in this respect, as suggested by the enhanced phagocytic killing of the transparent but not opaque variant of type 6B bacteria in the presence of increasing concentrations of CRP (72).

Activation of complement generates not only opsonins to facilitate phagocytosis but also the biologically active fragments C5a and C3a. This increases vascular permeability and activates mast cells to release inflammatory mediators. This attracts both polymorphonuclear leukocytes (PMNL) and monocytes to the site of infection which induce cytokine release from the phagocytes.

Whether complement is activated by classical, alternative, or the mannan-binding lectin pathway, by antibody, CRP, pneumococcal polysaccharide or lectins, the outcome is deposition of opsonising complement proteins (C3b, iC3b, or C4b) on the capsule, alongside specific IgG. Since phagocytic macrophages and PMNL constitutively express receptors for IgG (FcγRII, III) and complement components C3b, iC3b, or C4b (CR1, CR3), these molecules act in concert to facilitate phagocytosis, and are essential for pneumococcal clearance. The importance of opsonisation is strongly supported by the finding that the

impairment of either phagocytic system or opsonin production predisposes the host to pneumococcal disease.

Cell Death Proteins

These will include signalling molecules such as tumour necrosis factor alpha (TNF-α (73), Fas and Fas-associated death domain protein (FADD) as shown in Table 1.3 (74). These proteins can lead to apoptosis through activation of transmembrane death receptors such as Fas which causes receptors to aggregate together on the cell surface. This in turn activates the adaptor protein Fas-associated death domain protein (FADD) which then activates caspase-8, an initiator protein, to form a signal complex. This complex is now able to directly activate caspase-3, an effector protein, to initiate degradation of the cell. Active caspase-8 can also cleave BID protein to tBID, which acts as a signal on the membrane of mitochondria to facilitate the release of cytochrome c in the intrinsic pathway.

The mitochondrial stress pathway is initiated when a stress signal is activated, proapoptotic proteins in the cytoplasm, BAX and BID, stimulate the rupture of the mitochondria. The release of mitochondrial content is aided by the protein BAK. In the caspase dependant pathway, cytochrome c released from the mitochondria forms a complex in the cytoplasm with adenosine triphosphate (ATP) and apoptotic protease activating factor-1 (Apaf-1). This complex activates caspase-9, an initiator protein. In return, the activated caspase-9 works together with the complex of cytochrome c, ATP and Apaf-1 to form an apoptosome, which in turn activates caspase-3, the effector protein that initiates degradation (75).

The other cell death proteins include; caspase independent pathway (pyknosis) which is a result of apoptosis inducing factor (AIF). The

Table 1.3 Selected Proteins Directly Associated with Neuronal Cell Death

Protein	*Description*	*Action*	*References*
Cytochrome C (Cyt C)	A small heme protein found loosely associated with the inner membrane of the mitochondrion.	Cause ER calcium release leading to caspase 9 activation	(76)
Tumour necrosis factor (TNF-α)	TNF acts via the TNF Receptor (TNF-R) and is part of the extrinsic pathway for triggering apoptosis.	Associates with procaspases through adapter proteins (FADD, TRADD, etc.)	(77)
Caspases	Proteases, which exist as inactive proenzymes	Play essential roles in apoptosis (programmed cell death) and inflammation	(21)
Fas	Ligand which Associated with the Forms the Death Inducing Signalling Complex (DISC) upon ligand binding.	Fas pathway is sufficient to induce complete apoptosis in certain cell types through DISC assembly and subsequent caspase-8 activation	(74)
FADD	An adaptor molecule that bridges the Fas-receptor, and other death receptors, to caspase-8 through its death domain	Forms the death inducing signalling complex (DISC) during apoptosis	(74)
BAX	A pro-apoptotic member of the Bcl-2 protein family	Activated Bax forms an oligomeric pore in the outer membrane	(21)
Apoptosis inducing factor (AIF)	A flavoprotein found in the mitochondrial intermembrane space in healthy cells	Essential for nuclear disassembly in apoptotic cells	(21)
Creatine kinase BB (CKBB)	Catalyse the reversible transfer of a high-energy phosphate group between phosphocreatine (PCr) and ADP to maintain intracellular ATP levels in cells of high and fluctuating energy demands	Essential for storing, buffering and intracellular transport of "energy-rich" phosphate compounds in tissues with fluctuating high energy demand such as muscle, brain and other tissues and cells where CK is expressed	(44)

Proteins associated with the apoptotic pathway may be released into CSF. The levels of these proteins might increase during pneumococcal meningitis as a result of both the inflammatory response and the release of pneumococcal proteins. The table above shows proteins selected from the cell death cascade which have been identified in animal models of cell death and identified in the analyses of the CSF samples used in this study.

necrotic pathway is activated in severe meningitis. Alterations in the concentration of cytoplasmic calcium signal the mobilisation of executioner cathepsin proteases and other hydrolases, through calpain activation. Calpains have been implicated in the activation of pro-apoptotic caspase proteases; hence the later steps of necrosis correlate with the later steps of apoptosis.

1.4 Current Treatment

1.4.1 Antibiotic Treatment

Pneumococci can be killed with antibiotics such as ceftriaxone or cefotaxime. The choice of antibiotic depends on local patterns of epidemiology and patterns of resistance. The emergence of penicillin resistant isolates of pneumococci has hampered the use of β-lactam antibiotics for the treatment of pneumococcal infection. Monotherapy with penicillin can only be considered in areas with very low penicillin resistance rates (< 1%), although many experts recommend combination therapy for all patients (78).

In studies conducted in Egypt an association between mortality and penicillin resistance was reported by several studies at different time intervals, with an increase in the pattern of resistance over time (79).

In light of this emergence of penicillin-resistant strains (78), more advanced β-lactam antibiotics (cephalosporins) are commonly used in combination with other drugs such as vancomycin to treat meningitis (80). Delay in the initiation of antimicrobial therapy can result in poor outcome in this disease (4). A major problem with antibiotic therapy is that dead bacterial particulate matter can still stimulate inflammation. Steroid co-treatment is often used as a result.

1.4.2 Steroid Therapy

The benefit of steroid therapy is still not completely known. In developed nations, steroid adjuvant therapy has been shown to reduce deaths in some adults, particularly in patients with mild pneumococcal meningitis (81). However a large paediatric trial in Malawi demonstrated no benefit from steroids in children with bacterial meningitis (82).

1.4.3 Trial of Dexamethasone in Adults in Malawi

A double blind, randomised, placebo controlled trial of dexamethasone adjuvant therapy in adults with bacterial meningitis in Malawi showed no advantage after 40 days of treatment (2). The difference seen between Europe and Malawi is likely to have resulted from differences in the severity of the cases. There remains an urgent need for novel adjuvant therapy in the treatment on pneumococcal meningitis worldwide.

1.4.4 Pneumococcal vaccines for meningitis

Current pneumococcal vaccines elicit immune responses to cell-wall polysaccharides of pneumococci. The older 23-valent pneumococcal polysaccharide vaccine contains capsular polysaccharides of 23 serotypes responsible for about 90% of invasive pneumococcal infections (83). The vaccine is poorly immunogenic in certain groups who are at high risk for invasive pneumococcal infection, especially children younger than 2 years old (with relatively immature B cells), elderly people, and immunocompromised patients. The immune response in infants and young children can be improved by conjugation of pneumococcal polysaccharides to carrier proteins that enable activation of T cells, thereby enhancing antibody production and immunological memory (1). In regions where the 23-valent vaccine is used, administration of the vaccine is recommended early in the course of HIV disease.

Pneumococcal conjugate vaccines offer an alternative approach to preventing pneumococcal disease. The 7- and 9-valent conjugate vaccines have been shown to be highly efficacious. Such vaccines are effective at preventing invasive pneumococcal disease in HIV-infected children, although with a lower efficacy and duration of effect than in children without HIV infection (84;85).

1.4.5 Potential for New Therapy

Critical pathways for new therapy could potentially target the apoptotic and necrotic pathways. For example; citicholine has been shown to prevent neuronal damage when given before and after bacterial infection in animal models of meningitis regardless of the route of infection (86;87). Alternatively matrix metalloproteinase inhibitors could prevent blood brain barrier damage. Matrix metalloproteinase's (MMP) are a family of zinc-dependent endopeptidases that show affinity to different components of the extracellular matrix. They have been shown to play a role in the breakdown of the blood-brain barrier and the facilitation of neuroinflammation in bacterial meningitis (88). In bacterial meningitis MMPs may contribute to the development of brain injury by both their proteolytic activity on the extracellular matrix and their ability to increase the levels of soluble TNF-α, a pivotal element in the meningeal inflammatory process. TNF-α is a strong stimulus for the release and activation of MMPs in the brain (89;90).

1.4.6 Critical Gaps in Knowledge

There are critical gaps in knowledge that need to be addressed before new therapies can be implemented in meningitis. In particular there is insufficient data to link human death in meningitis with the mechanisms observed in animal models. High levels of CSF apoptosis proteins in patients with neurological damage or death would provide a basis for trials of citicholine. Alternatively, high levels of MMP and

MMP-related damage would provide a case for the use of MMP inhibitors. Proteomics methods provide a modern means of obtaining these pivotal data.

1.5 Current Genomic Data Available for the Pneumococcus

The complete genome sequence of a capsular serotype 4 isolate of *S. pneumoniae* [designated TIGR4; TIGR indicates The Institute for Genomic Research] was determined by the random shotgun sequencing strategy (Gen- Bank accession number AE005672; see www. tigr.org/tigr-scripts/CMR2/CMRHomePage. spl). The 2,160,837 – base pair genome sequence of this pneumococcal isolate contains 2236 predicted coding regions; of these, 1440 (64%) were assigned a biological role. Approximately 5% of the genome is composed of insertion sequences that may contribute to genome rearrangements through uptake of foreign DNA. Several surface-exposed proteins that may serve as potential vaccine candidates were identified. Comparative genome hybridization with DNA arrays revealed strain differences in *S. pneumoniae* that could contribute to differences in virulence and antigenicity (91).

There are nine further *S. pneumoniae* genomes available for analysis, although some of these are in the process of completion. Information generated form the comparative analysis of these sequences will provide insights into the evolution of these genomes, and will serve as a building block to understand the complexity of a species such as the Pneumococcus (92).

1.6 The Proteome and Proteomics

The proteome refers to the entire quota of protein expressed by the genome of an organism or cell type. This quota of proteins varies with

time. Proteomics is the quantitative analysis of expressed proteins present in cells, tissues or organisms at a certain time and under different conditions; like genomics, it takes a global, system-wide view of the organism (93). Serum proteomics provide a pattern-based diagnosis, because multiple peptide and protein signals are detected simultaneously in a single mass spectrum. Furthermore, disease states seem to be characterised by raised levels of cleavage products of abundant serum proteins (94).

1.6.1 Application of Proteomics to Pathogenic Diseases

Proteomics is a technique which has previously been applied to different pathogenic diseases with successful outcomes. Rao *et al* used proteomics to study host immune response to *Pseudomonas aeruginosa* in patients with cystic fibrosis (95). In this study proteomics was applied to serum. It was observed that the outer membrane protein L (OprL), a non-type III secretion system protein, were an early immunogenic protein during the initial *P. aeruginosa* infection of patients with cystic fibrosis.

Another study with a successful outcome from proteomic analysis was from Agranoff *et al* (96). In this study, sera were studied from patients with active tuberculosis with proteomics. They were able to discriminate the proteomic profile of patients with active tuberculosis from that of controls with overlapping clinical features.

1.6.2 Cerebrospinal Fluid Proteomics

Cerebrospinal Fluid

Cerebrospinal fluid is a clear bodily fluid which resides in the subchranoid space between the arachnoid and pia mater layers of the meninges. It is a saline solution with microglia and one of its main actions is to act as a buffer for the cortex. It is produced by specialised ependymal cells of the choroid plexus located within the brain (97). CSF

thus provides an ideal proteome for qualitatively comparing the quota of proteins present in CSF as a consequence of meningitis, leakage of the blood brain barrier or from bacteria.

Background to Patient CSF Used In This Thesis

CSF used in this thesis was collected as part of a clinical trial into the use of corticosteroids for the treatment of bacterial meningitis in adults (2). As part of this study CSF was collected by lumbar puncture from 465 patients. A second lumbar puncture was taken from patients whom survived after 48hrs if a) they were conscious and able to consent or b) there was a clinical imperative to repeat the LP (such as deterioration or failure to improve despite appropriate therapy, diagnostic uncertainty, intracranial pressure control). The majority of these patients also had HIV co-infection. Patients were randomly assigned to receive dexamethasone (233 patients) or placebo (232 patients) plus intramuscular ceftriaxone (230 patients) or intravenous ceftriaxone (235 patients). The study design and data collection were conducted by members of the University of Malawi, College of Medicine.

1.7 Proteomic Methods Applicable to CSF Analysis

Proteomic techniques applicable to CSF are either a "shotgun" approach (complex peptide mixtures from multiple proteins are analysed with mass spectrometry at once) or the identification of specific proteins, extracted from polyacrylamide gels, from the peptide spectra of digested protein based off the characteristic peptide spectra of that protein (a peptide mass fingerprint, PMF) (98;99). Protein identification in this thesis utilised the latter method for the analysis of proteins in the CSF samples obtained from the clinical trial of dexamethasone described previously (2).

The applications of proteomics to CSF samples have allowed the presence or absence of proteins to be associated with neurological damage and death in meningitis. Proteomics approaches allow the analysis of a large spectrum of host and pathogen proteins but cannot yet be applied to the bacterial cell wall components such as lipoteichioic acid (LTA) of pneumococcus.

As discussed, these methods have already been applied in malaria and tuberculosis. This thesis is the first application of proteomics to the investigation of pneumococcal meningitis (58;100). In this thesis we have used a number of proteomic techniques to analyse CSF. The rationale for using these techniques are discussed below and the detailed methodology in the methods section. In support of the use of proteomics, add something to indicate that this technology has been use successfully with other bacteria (a summary table, or at least a brief paragraph with a few references).

1.7.1 The Bradford Assay

To account for variability in the amount of protein loaded, protein concentration was determined using the Bradford assay. The Bradford Assay is a dye-binding assay in which a differential colour change of a dye occurs in response to various concentrations of protein. The absorbance maximum for an acidic solution of Coomassie® Brilliant Blue G-250 dye shifts from 465 nm to 595 nm when binding to protein occurs. The Coomassie blue dye binds to primarily basic and aromatic amino acid residues, especially arginine (101).

The advantages of the Bradford assay include; ease of use, sensitivity and low cost of reagents. Also for microplate-based assays the reagent volumes can be decreased to a volume as low as 10 µl. This was

useful in preserving the CSF samples whilst obtaining a reproducible protein concentration for use in the analysis.

The accuracy of quantification with Bradford reagent is dependent on the amino acid sequence of the protein (101). Thus a suitable positive control/calibration protein was needed. Bovine serum albumin was used as the positive control. However it has been noted to give low protein concentrations. This had no effect on the outcome of any experiments undertaken in this thesis as all expression data was normalised. Thus the method and the amount of protein loaded were kept constant for all experimental analyses.

1.7.2 Polyacrylamide Gel Electrophoresis (PAGE) of Proteins

Gel Electrophoresis

Gel electrophoresis is a technique that separates macromolecules on the basis of size, electric charge, and other physical properties (102). In gel electrophoresis proteins are separated according to molecular weight. Polyacrylamide acts as a molecular sieve allowing smaller proteins to reach the bottom of the gel before the larger proteins. Protein on polyacrylamide gels can then be visualised by staining or transferred to a membrane (Western blot).

Protein Denaturation and Solubilisation

Protein denaturation is essential to separate proteins. Before separating the proteins, they are treated with sodium dodecyl sulfate (SDS). Protein denaturation allows attachment of a number of SDS molecules roughly proportional to the protein's length. A protein's length when unfolded is roughly proportional to its mass. This is equivalent to saying that it attaches a number of SDS molecules roughly proportional to the protein's mass. Since the SDS molecules are negatively charged all of the proteins will have approximately the same mass-to-charge ratio as

each other. The problem imposed by this compound is that the negative charge can affect the movement of the protein within the gel. This was counteracted by the addition of the zwitterionic detergent: 3-[(3-chlor-amidopropyl)-dimethyl ammonio-1-propane sulfonate (CHAPS).

Other denaturing agents, such as iodoacetamide, help break down disulphide bonding within the protein. In addition a high concentration of urea and the reducing agent dithiothreitol was used in order to further denature the proteins. These compounds also aid solubilisation of certain types of protein, in particular hydrophobic proteins, which can affect the results of the gel separation.

2D PAGE

The technique of 2-dimensional PAGE is a modified form of SDS-PAGE in which separation of macromolecules depends upon two forces: charge and mass (103). The first dimension separates protein molecules according to protein isoelectric point (pI). Proteins can carry positive, negative or zero charge depending on their local pH, and for every protein there is a specific pH at which its net charge is zero; this is its pI.

Commercial immobilized pH gradient (IPG) strips improve the resolution of 2D PAGE and allow a higher level of sample load (104). Protein pI's generally fall in the pH range 3 - 12, with most being around 4 - 7. In this analysis the majority or proteins fell within the pH 4 – 7 region (105).

The second dimension separates proteins based on their molecular weight. The second dimension is usually performed using a standard SDS-polyacrylamide gel. Here an electric potential is again applied, but at a 90° angle from the first field. The proteins are attracted to the

negative side of the gel proportionally via their mass-to-charge ratio (103).

Staining

Standard SDS-PAGE gels were stained using colloidal Coomassie blue (CBB) stain and 2D gels were stained using silver stain. Colloidal Coomassie blue stain can detect protein quantities up to 100 ng.

The use of silver staining allowed protein quantities as low as 5-7 ng to be detected (106). The silver nitrate bonds to cysteine groups within the protein. The silver is darkened by exposure to ultra-violet light. The darkness of the silver can be related to the amount of silver and therefore the amount of protein at a given location on the gel (107).

Excessive staining effects can be a problem with the method but this has been resolved using computational methods for gel matching as spot normalisation will remove most of this effect as discussed in chapter 3 (108).

1.7.3 Mass Spectrometry

Mass spectrometry is a powerful analytical technique which is used to analyse small molecules and complex biomolecules, such as DNA, lipids, carbohydrates and proteins. The technique involves the study of the mass-to-charge ratio (m/z) of atoms, molecules or fragments of molecular ions. Mass spectrometry is a destructive method of analysis as it forms and utilises ions rather than molecules thus conversion back to the starting material is impossible (109). All mass spectrometers have the same 4 sections; an inlet system; an ion source; a mass analyser; and a detector.

Mass spectrometry only works for charged species and in order to obtain a mass spectrum, gaseous molecules or species desorbed from

condensed phases must first be ionised. There are various techniques available for ionisation depending on the ion source. Direct ion sources exist as two types: liquid-phase ion sources and solid-state ion sources. Depending on the method of ionisation there are different inlet systems available. The purpose of the inlet system is to permit introduction of a representative sample into the ion source with a minimal loss of pressure.

Once ions have been generated they are separated according to their mass-to-charge ratio using a mass analyser. Instruments with several analysers in sequence are used for tandem mass spectrometry (MS/MS) (110). There are three main characteristics of a mass analyser: the upper mass limit, the transmission and the resolution. The mass limit determines the highest value of m/z (in Th or atomic mass units (amu), where z = 1) that can be measured. Transmission is the ratio between the number of ions reaching the detector and the number of ions produced in the source. Resolving power is the ability to produce distinct signals for two ions with a small mass difference.

MALDI-TOF

MALDI Ionisation

Matrix assisted laser desorption/ionisation-time of flight (MALDI-TOF) is a mass spectrometry method utilising a solid-state ion source in which the analyte is in an involatile deposit (a matrix). In this analysis the matrix used was alpha-cyanao-4-hydroxycinnamic acid. The peptide digest mixture molecules are embedded throughout the matrix (isolated from each other). Bulk portions of the solid solution were ablated by intense pulses of laser for a short duration. This induces rapid heating of crystals through excitation of matrix molecules causing localised sublimation and expansion into the gas phase. Ionisation of the analyte may occur at any time during this process (111).

Three different types of fragmentation in MALDI can occur; fragmentation at the sample surface (prompt fragmentation), fragmentation in the source, after desorption, but before acceleration (fast fragmentation) and after acceleration (post-source decay, PSD fragmentation) (112). The first two are always apparent in MALDI spectra; the last method is only seen under certain conditions.

Time of Flight Mass Analyser

MALDI utilises a Time-of-flight (TOF) mass analyser to detect the short-lived ions signals. Positive ions that are formed are accelerated by an electric field pulse which has a similar frequency but slightly lags behind the ionisation pulse.

Accelerated ions pass into a region containing no external field known as a drift tube. Assuming all ions have the same kinetic energy then their velocities will depend upon their masses. Heavier ions will arrive at the detector up to 30 μs after lighter ones (113). The detector is set up so that we may have an instantaneous display of the mass spectrum. Time resolution increases with increasing distance, d, and deceases with an increase in voltage.

In principle, upper mass range of TOF has no limit, which is very good for soft ionisation techniques. These instruments also have high transmission efficiency, which leads to high sensitivity *e.g.* 100-200 amol of cytochrome c can be observed. In principle, all ions formed are analysed (114).

One major drawback of TOF is poor mass resolution caused by factors including; length of ion formation pulse (time distribution), size of volume where ions are formed (space distribution), and variation in kinetic energy of ions (KE distribution). Mass resolution is improved by

using an electrostatic reflector, known as a reflectron. The reflectron creates a retarding field that acts as an ion mirror. It deflects ions and sends them back through the flight tube effectively increasing d. The term reflectron TOF is used to differentiate this method from a linear TOF. The reflectron corrects for KE dispersion at the reflecting point, increasing mass resolution. However, there is a loss of sensitivity and also the mass range is limited to maximum of 3500 Da.

The spectra generated from MALDI-TOF provide a peptide mass fingerprint (PMF) of the protein as shown in figure 1.4. The peptide sequences can be searched *in silico* using online protein databases (94).

Liquid Chromatography with Tandem Mass Spectrometry (LC-MS/MS)

This method of mass spectrometry involves electrospray ionisation. This method of ionisation utilises a liquid-phase ion source. The peptide mixture is in solution and the sample is directly injected into the mass spectrometer via a liquid chromatography machine. This solution is introduced, by nebulisation, as droplets into the mass spectrometer.

Liquid Chromatography (LC)

In Liquid Chromatography a liquid sample is applied to a narrow cross section of a column which is filled with a particular packing. A solvent is passed through a column sweeping the sample from the inlet to the outlet.

The mobile phase is typically a solvent moving through the column which carries the mixture to be separated (115;116). The mobile phase can be adjusted to improve the separation; solvents can be changed as well as their ratios.

The stationary phase is located within a column. Different stationary phases may be employed such as; normal (or adsorption/displacement),

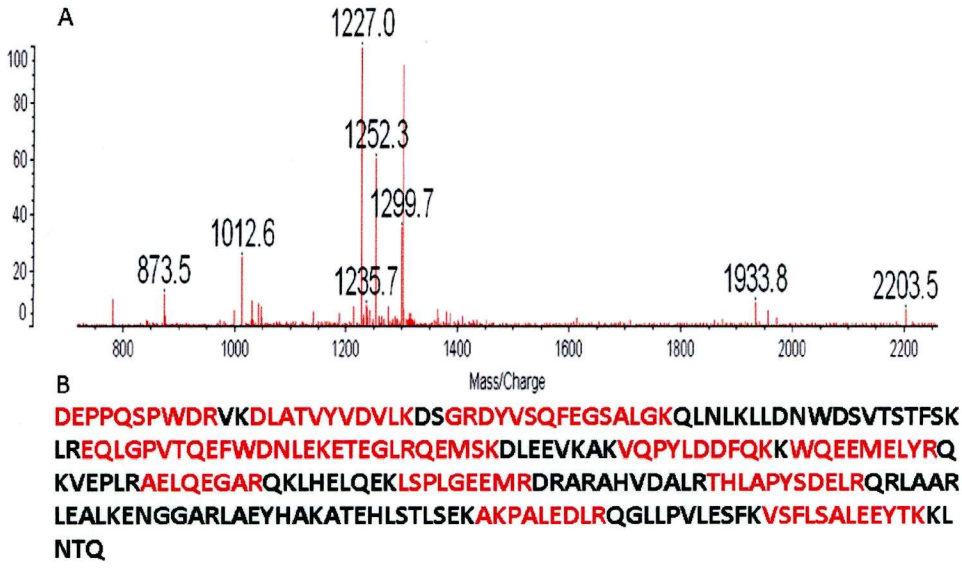

Figure 1.4 Representative PMF generated by MALDI-TOF. ***A*** *Representative PMF mass spectrum generated by MALDI-TOF mass spectrometry.* ***B*** *Peaks correspond to specific peptides shown in red.*

reversed (lipophilic), ion exchange, size exclusion or bioaffinity. The phases are chosen such that components of the sample have differing solubility's in each phase. A component which is quite soluble in the stationary phase will take longer to travel through it than a component which is not very soluble in the stationary phase but very soluble in the mobile phase. As a result of these differences in mobility's sample components will become separated from each other as they travel through the stationary phase (115;116).

There are many different kinds of detectors available for LC and most commonly they use the optical properties of the analytes e.g. absorption (UV/Vis detectors) or fluorescence (spectrofluorometric detectors).

Electrospray Ionisation

Along with MALDI the other major ionisation technique for biomolecular analysis is electrospray ionisation (ESI). ESI is an atmospheric pressure ionisation source. ESI is important for biomolecular analysis (such as proteins and oligonucleotides) as they can be analysed using mass analysers with a relatively low m/z range. ESI allows very high sensitivity to be reached and is easily coupled to LC (114).

An electrospray is produced by applying a strong electric field under atmospheric pressure to a liquid passing through a capillary tube with a weak flow (typically 1 – 10 $\mu l min^{-1}$). The Field induces charge accumulation at the surface of the liquid at the end of the capillary. Liquid breaks to form highly charged droplets. The Spray starts at a voltage (onset voltage) that for a given source depends upon the surface tension of the solvent (114) with formation of a "Taylor cone" (117) as shown in figure 1.5. Solvent contained in the droplets evaporate causing them to shrink to a point where repelling coulombic forces (the

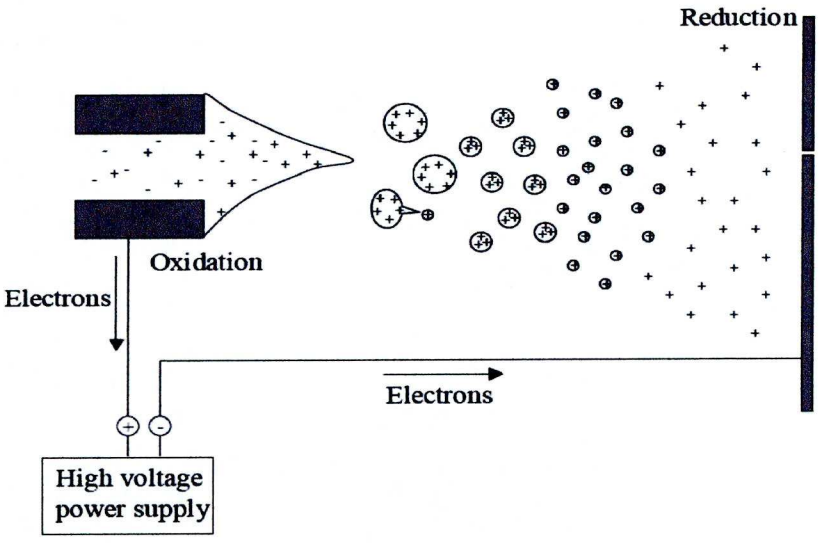

Figure 1.5 ESI ion formation. *Generation of ions during electrospray ionisation with generation of a Taylor cone.*

electrostatic interaction between electrically charged particles) are close to cohesion forces. Due to the strength of the field a cascade of ruptures occur generating highly charged droplets. When the electric field at the surface is large enough desorption of ions occurs. Desorption of charged molecules occurs from the surface thus sensitivity is higher for compounds formed in higher concentrations at the surface i.e. more lipophilic.

ESI is sensitive to concentration rather than total amount of sample. Reducing flow rates (thus increasing concentration) increases sensitivity. At the lower limit this is nanolitre per min (nl min^{-1}, nanoelectrospray, nESI) as used in this analysis (118).

Ion Trap Mass Analyser

The mass analyser used in this analysis was an ion trap mass analyser. An ion trap is a combination of electric or magnetic fields that captures ions in a region of a vacuum system or tube. Ions with different masses are present together within the ion-trap and then expelled according to their masses to obtain spectrum. As ions in the trap expel each other, their trajectories expand as a function of time. Following injection ions of different mass are stored together. By applying a RF voltage of selected frequency and variable amplitude it is possible to select ions according to their m/z. Tandem MS was time-dependent in the trap. A sequence of events takes place to allow MS^n. Ions of one m/z are selected by expelling all the others from the ion-trap.

MS/MS spectra are informative about the composition and the order of amino acids in a peptide sequence as it can reveal the molecular weights of the peptide's breakdowns. Several bonds along the backbone of a peptide can be induced by collisions. If the charge is retained on the N-terminal fragment, the ion is classified as a, b or c, and if the charge is

retained on the C-terminal, the ion is classified as x, y or z as shown in figure 1.6. It has been observed that in a typical MS/MS spectral dataset, the majority of the N- and C-terminal ions are b and y ions, respectively, and each of these ion types contains the derivatives of neutral loss of water or ammonia (119).

1.8 Mascot

Mascot is a powerful search engine that uses mass spectrometry data to identify proteins from primary sequence databases. This search engine uses the MOWSE statistical algorithm to confirm the identity of proteins from peptide mass spectrometry data.

1.8.1 Mowse Probability Score

Peptide mass fingerprints and MS/MS fingerprints have been used to confirm protein sequences allowing the detection of translation errors, mutation or insertion, characterize post-translational modifications or processing events, and assign disulphide bonds. Such peptide-mass information can provide a distinctive fingerprint signature, which is sufficiently discriminating to allow the unique and rapid identification of unknown sample proteins. The Mowse scoring algorithm was developed by Pappin in 1993 (120). Mowse databases comprise calculated molecular weights of all peptide fragments derived from a set of specific enzyme or reagent cleavage rules. The first stage of a Mowse search is to compare the calculated peptide masses for each entry in the sequence database with the set of experimental data. Mascot incorporates a probability based implementation of the Mowse algorithm. Rather than just counting the number of matching peptides Mowse uses empirically determined factors to assign a statistical weight to each individual peptide match.

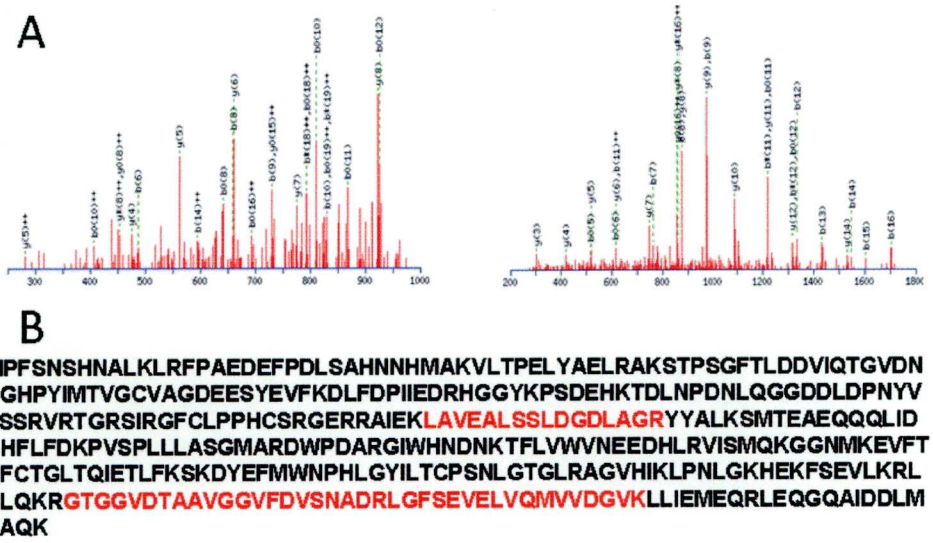

Figure 1.6 Representative spectra generated by MS/MS. ***A*** *Representative MS/MS spectra generated by peptide spectra.* ***B*** *This spectra is analysed against protein databases which are represented by red sequences.*

1.8.2 Important Search Fields

Database

In this analysis SwissProt or MSDB databases were used to analyse the PMF and MS/MS data because of the high quality of the protein sequence data. The databases available on this Mascot are shown in table 1.4.

Taxonomy

The taxonomy of the species under analysis can be changed to include all species or specific species. Analysis of smaller genomic organism's results in an increase in the possibility of false positives therefore it is necessary to increase the number of searches to avoid the possibility of false positives. In this analysis the spectra were compared to *Homo sapiens*, *Streptococcus pneumoniae*, all mammalian, all bacteria and all species.

Enzyme

As a result of the varying cleavage specificity of the enzymes used for digestion as shown in table 1.5 it was necessary to specify the reagent used for protein digestion. Trypsin was used in all digestion experiments.

Modifications

Mascot supports two types of modification; fixed modifications are applied universally, to every instance of the specified residue(s) or terminus. There is no computational overhead associated with a fixed modification it is simply equivalent to using a different mass for the modified residue(s) or terminus e.g. selecting carboxymethyl (C) means that all calculations will use 161 Da as the mass of cysteine.

Variable modifications are those which may or may not be present. Mascot tests all possible arrangements of variable modifications to find

Table 1.4 Non-redundant Databases Available on Mascot

Database	*Comment*
EST	EST divisions of Genbank, (currently EST_human, EST_mouse, EST_others)
MSDB	Comprehensive, non-identical protein database
NCBI nr	Comprehensive, non-identical protein database
SwissProt	High quality, curated protein database

SwissProt and MSDB were mostly used in this thesis for their high quality protein databases.

Table 1.5 Enzyme Reagents Which Can Used with Mascot

Enzyme Name	*Cleave*	*Don't cleave*	
Trypsin	KR	P	CTERM
Arg-C	R	P	CTERM
Asp-N	BD		NTERM
Asp-N_ambic	DE		NTERM
Chymotrypsin	FYWL	P	CTERM
CNBr	M		CTERM
Formic_acid	D		CTERM
Lys-C	K	P	CTERM
Lys-C/P	K		CTERM
PepsinA	FL		CTERM
Tryp-CNBr	KRM	P	CTERM
TrypChymo	FYWLKR	P	CTERM
Trypsin/P	KR		CTERM
V8-DE	BDEZ	P	CTERM
V8-E	EZ	P	CTERM
CNBr+Trypsin	M KR	P	CTERM
None	-	-	-
Semitrypsin	-	-	-

The enzyme used for this analysis was trypsin. Trypsin cleaves between arginine and lysine except when next to proline.

the best match e.g. if oxidation (M) is selected and a peptide contains 3 methionines. Mascot will test for a match with the experimental data for that peptide containing 0, 1, 2, or 3 oxidised methionine residues.

Variable modifications can be a very powerful means of finding a match, but there are also dangers to be aware of. Even a single variable modification will generate many possible additional peptides to be tested. More than one variable modification causes the number of arrangements to increase geometrically. This means that a search can take dramatically longer than the same search with fixed modifications. More importantly, testing all possible arrangements of modifications generates many more random matches, so that discrimination can be sharply reduced.

Peptide and MS/MS Tolerance (±)

The peptide tolerance is the error window on experimental peptide mass values which takes into account isotopic variation. The MS/MS tolerance is error window for MS/MS fragment ion mass values.

Mass Values

Specifies whether experimental peptide mass values in a peptide mass fingerprint search include the mass of the charge carrier, MH^+ or M^-H^-, or whether they correspond to a neutral Mr value.

Peptide Charge

The peptide charge is used to specify the precursor peptide charge state in a sequence query or an MS/MS ions search. The peptide mass value supplied in an MS/MS data file is usually an observed m/z value. The charge state field is used to calculate the relative molecular mass (Mr) of the precursor from the observed m/z unless the data file explicitly specifies a different charge state.

Missed Cleavages

Setting the number of allowed missed cleavage sites to zero simulates a limited digest. This setting assumes the digest is perfect with no partial fragments present. This will give maximum discrimination and the highest score.

Digest mixtures usually include partial digestion i.e. peptides with missed cleavage sites. Therefore a setting of 1 was chosen. A higher level of missed cleavages increases the number of calculated peptide masses to be matched against the experimental data. If the actual digest does not contain extended partials, this simply increases the number of random matches, and so reduces discrimination.

Instrument

Variations in the mass analyser effect which fragment ion series will be used for scoring. For ion trap mass analysers the standard fragments include; 1^+ fragments, 2^+ fragments (if precursor 2^+ or higher), b series ions, b-NH_3 (if fragment includes RKNQ), b-H_2O (if fragment includes STED), y series ions, y-NH_3 (if fragment includes RKNQ) and y-H_2O (if fragment includes STED).

1.9 Gene Ontology

Gene Ontology (GO) provides a structured, precisely defined, common, controlled vocabulary for describing the roles of genes and gene products in any organism (121). The vocabulary consists of three groups; molecular function, biological process and cellular component.

1.9.1 Molecular Function

GO molecular function terms represent activities rather than the entities (molecules or complexes) that perform the actions, and do not specify where or when, or in what context, the action takes place.

Molecular functions generally correspond to activities that can be performed by individual gene products, but some activities are performed by assembled complexes of gene products.

1.9.2 Biological Process

GO biological process is a series of events accomplished by one or more ordered assemblies of molecular functions. It can be difficult to distinguish between a biological process and a molecular function, but the general rule is that a process must have more than one distinct step.

1.9.3 Cellular Component

GO cellular component refers to the place in the cell where a gene product is active.

1.10 ClustalW

ClustalW is a general purpose multiple sequence alignment program for DNA or proteins. It produces biologically meaningful multiple sequence alignments of divergent sequences. It calculates the best match for the selected sequences and lines them up so that the identities, similarities and differences can be seen. Evolutionary relationships can be seen via Phylograms. The basic information they provide is very useful in designing experiments to test and modify the function of specific proteins, in predicting the function and structure of proteins and in identifying new members of protein families (122)

ClustalW uses a computer generated pairwise alignment of protein sequences. Using the alignments of the protein sequences being compared an evolutionary distance is computed. This distance is calculated by looking at the non-gapped positions and the number of mismatches between the two sequences. This value is then divided by the number of non-gapped pairs to calculate the distance. Once all distances

for all pairs are calculated they go into a matrix. ClustalW constructs a similarity tree using this matrix and Neighbour-Joining.

1.11 Western Blot

The Western blot (immunoblot) is an analytical technique used to detect specific proteins in a given sample of tissue homogenate or extract. Protein separated using SDS PAGE was transferred to a membrane made of nitrocellulose or Polyvinylidene Fluoride (PVDF). The membranes are probed using antibodies specific to the target protein. Nitrocellulose and PVDF are used for immobilisation of proteins in Western blots for their non-specific affinity for amino acids.

1.11.1 Western Blot Image Development

Alkaline phosphatase (AP) and horseradish peroxidase (HRP) are the two enzymes used most extensively as labels for protein detection. An array of chromogenic, fluorogenic and chemiluminescent substrates are available for use with either enzyme. Alkaline phosphatase offers a distinct advantage over other enzymes in that its reaction rate remains linear allowing sensitivity to be improved by simply allowing a reaction to proceed for a longer time period. Unfortunately, the increased reaction time often leads to high background signal resulting in low signal:noise ratios. HRP conjugated antibodies are considered superior to antibody-AP conjugates with respect to the specific activities of both the enzyme and antibody due the smaller size of HRP enzyme and compatibility with conjugation reactions. In addition the high activity rate, good stability, low cost and wide availability of substrate makes HRP the enzyme of choice for most applications.

BCIP/NBT reacts with alkaline phosphatase forming Formazan as an insoluble purple precipitate. DAB tetrahydrchloride reacts with HRP in the presence of hydrogen peroxide (H_2O_2) forming DAB as an

insoluble brown precipitate. ECL reacts with HRP in the presence of H_2O_2 to form an unstable compound which emits chemiluminescence. Detection may be documented with x-ray film. Of these detection methods, x-ray film is the most sensitive method.

1.12 Thesis Aims and Project Hypothesis

The aims of this thesis are to (1) compare the 2D PAGE pattern of proteins found in the CSF obtained at admission from groups of patients with pneumococcal meningitis in Malawi. (2) Formally identify proteins found to be distinguishable between non-survivors and survivors using mass spectrometry. (3) Relate these proteins to known mechanisms of pneumococcal meningitis in order to generate hypotheses. (4) Validate the identity of selected proteins from the list obtained and confirm the expression of specific proteins from host and pneumococci present in CSF.

This thesis will test the general hypotheses; (1) Proteins present in CSF of patients with pneumococcal meningitis are different to those proteins present in normal CSF. (2) Host and pneumococcal proteins in CSF have an association with clinical outcome.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Introduction

The aim of this chapter is to provide information on the samples used and on the experimental techniques which have been utilised to provide the information in this thesis. This chapter will provide a comprehensive step-by-step methodology of the general techniques used in this thesis allowing easy reproducibility.

2.2 Patient Information

2.2.1 Background of Patients and CSF samples

CSF used for the experimental analysis was chosen from patients who had an excellent recovery and short clinical course. These subjects all survived with no neurological impairment (median duration of symptoms etc).

As discussed in the introduction patients were recruited to a double blind, randomised, placebo controlled trial of dexamethasone adjuvant therapy. The study was conducted in adults with bacterial meningitis presenting at the Queen Elizabeth Central Hospital in Blantyre, Malawi between May 2002 and Jan 2005 in (2). The study was approved by the research ethics committees of the University Of Malawi College Of Medicine and the Liverpool School of Tropical Medicine. Patients or their legal guardians provided written informed consent or, if they were unable to read or write, they provided independently witnessed verbal consent before recruitment. Patients were treated in hospital for a minimum of 10 days and were evaluated at 40 days and at 6 months. Clinically evident adverse events were recorded systematically throughout the trial period. At follow-up, patients had a standardized neurologic examination and a hearing assessment. Patients who did not return for follow-up appointments were visited at home. No other

underlying diseases were specifically looked for other than HIV and malaria. However, some patients volunteered a past medical history. Patients in Malawi have basic medical care as described previously (2).

The eventual cause of death was often not known. However we have initiated a new study (2010 – 2012) to determine causes of death in a new prospective cohort.

2.2.2 Patient Categories

Of those who survived, 103 patients survived to one month with no neurological impairment or recorded disability, 66 survivors had detectable hearing loss without complete deafness and 47 patients had disability (paresis, intellectual impairment, blindness, complete deafness debility or recurrent seizures). 249 of these patients died, of which 92 survived for 10 days or more but died before 40 days from presentation, and 157 patients died within 10 days of presentation to hospital. Initial group definitions included: Survival with no neurological impairment or recorded disability (n = 103); deaf (n = 66); maimed (n = 47); protracted death (n = 92); rapid death (n = 157). Recruitment included consent for diagnostic samples to be used in meningitis pathogenesis research at a later date.

2.2.3 Diagnosis

Probable bacterial meningitis was defined as a cerebrospinal fluid specimen containing a cell count of more than 100 per cubic millimetre with more than 50% neutrophils without identification of an organism (See appendix A). Recruitment included consent for diagnostic samples to be used in meningitis pathogenesis research at a later date. CSF specimens were examined as previously described (2). CSF was examined by means of microscopy for cell and differential counts. A

Gram's stain procedure was performed if the sample was turbid or had more than 10 white cells per cubic millimeter. Centrifuged CSF deposits were incubated on sheep-blood agar in a candle- extinction jar at 37°C for 48 hours and isolates were identified by means of standard techniques. Blood was cultured at 37°C for a minimum of 48 hours (BacT/Alert, bioMérieux). In the first 51 consecutive CSF specimens for which Gram's stain and culture were negative, polymerase chain reaction (PCR) for meningococcus and pneumococcus was performed (2).

Proven bacterial meningitis was defined as identification of an organism from a CSF specimen by means of microscopy, culture, or PCR or from blood culture in the context of a CSF specimen containing a white-cell count of more than 100 per cubic millimeter with more than 50% neutrophils. Probable bacterial meningitis was defined as a CSF specimen containing a cell count of more than 100 per cubic millimeter with more than 50% neutrophils without identification of an organism (2).

All subjects were HIV positive and all samples were collected before any treatment commenced. The median CD4 cell count, on admission for 101 consecutively admitted HIV-positive patients during recruitment to the trial was 102 per cubic millimeter (interquartile range, 51 to 169). We did not measure CD4 counts on all but one of these patients as anti-retrovirals were not routinely available until mid 2004 in Malawi and therefore there was no clinical imperative and no advantage to the patient to know their CD4 count. None of these patients were on anti-retroviral therapy. All had WHO stage III or IV disease (1990 criteria). As described previously normal CSF was obtained from patients who tested negative for meningitis or any other pathogen. The HIV status of the normal patients was not known.

2.3 General Solutions and Reagents

All chemicals were purchased from Sigma Aldrich (Poole, UK), VWR (Lutterworth, UK) or Fisher (Loughborough, UK) unless otherwise stated. General solutions used for the analysis were made using HPLC grade water. The pH of all solutions used in this thesis was adjusted by the addition of 1 M HCl or NaOH. Solutions used in thesis are presented in appendix B.

2.4 The Bradford Assay

The concentration of protein was determined using the Bradford assay. Samples were analysed in triplicate on a standard 96-well plate. The calibration standard used for the analysis was BSA. If the coefficient of variation between the replicates was > 5% the assay was repeated. Protein concentrations were rejected if the BSA standard curve did not have an R^2 value of >0.98.

2.5 SDS Polyacrylamide Gel Electrophoresis (PAGE) of proteins

2.5.1 Gel Electrophoresis

SDS PAGE was undertaken using the PROTEAN II gel electrophoresis kit (BioRad). The concentration of gels used for the separation of proteins is shown in table 2.1. The proteins were separated in SDS supplemented tank buffer.

2.5.2 2D PAGE

This variation of SDS PAGE is discussed in detail in chapter 3.

2.5.3 Staining

SDS PAGE gels were stained using colloidal coomassie blue (CBB) stain. In this method of staining gels were incubated overnight in CBB.

Table 2.1 Gel Concentrations used for this analysis

Reagents	***Gel concentrations***				
	5%	***7.5%***	***10%***	***12.5%***	***15%***
30% Acrylamide/bisphosphate	0.83 ml	1.25 ml	1.7 ml	2.0 ml	2.5 ml
Trizma base pH 8.8	1.3 ml	1.3 ml	1.3 ml	1.3 ml	1.3 ml
10% SDS	50 µl	50 µl	50 µl	50 µl	50 µl
Ultra-pure H_2O	2.77 ml	2.35 ml	1.9 ml	1.6 ml	1.1 ml
10% APS	50 µl	50 µl	50 µl	50 µl	50 µl
TEMED	2 µl	2 µl	2 µl	2 µl	2 µl

The table above shows the quantity of reagents required to prepare the gel concentration required for separation of protein by SDS PAGE.

Gels were washed the following day in destaining CBB wash 1 for 1 min followed by destaining solution for 2 hrs at RT.

2D gels were stained using silver stain. The use of silver staining allowed protein quantities as low as 5-7 ng to be detected (106). Gels were fixed overnight in fixing solution. Fixing solution was washed off using distilled water for 30 mins. Gels were then incubated in 0.03% (w/v) sodium hydrosulphite for 2 mins followed by washes in distilled water. Gels were then incubated in silver nitrate solution for 40 mins before washing with distilled water. Spot images were developed by the addition of silver developing solution for 7-12 min. The reaction was stopped using silver stopping solution for 2 hrs at RT.

The silver nitrate bonds to cysteine groups within the protein. The silver is darkened by exposure to ultra-violet light. The darkness of the silver can be related to the amount of silver and therefore the amount of protein at a given location on the gel (107). Excessive staining effects have been surmounted using computational methods for gel matching as spot normalisation will remove most of this effect (108).

2.5.4 2D Gel Comparison

This is discussed in detail in chapter 3.

2.5.5 Gel Excision

Following the selection of the spots of interest, they were manually excised from the stained gels and subjected to in-gel tryptic digestion (123).

Protein spots of interest were manually excised from the gel using pen picking. Silver stained samples were destained using a 1:1 mix of 100mM sodium thiosulphate and 30 mM potassium ferracyanide. Spots were equilibrated in 200 mM ammonium bicarbonate (ambic) and

dehydrated using a 2:1 acetonitrile (ACN):ambic (25 mM) solution. Spots were vacuum centrifuged to dryness and a 12.5 ng/µl solution of sequencing grade, modified trypsin (Promega, Madison, WI, USA) in ambic (25 mM) was applied to the gel spot. In-gel digestion was carried out overnight at 37°C. Peptide extraction was carried out by sonication (10 min) followed by centrifugation (10000 RPM) and transfer of supernatant to a new microcentrifuge tube. Neat ACN was added to the gel spot and incubated for a further 30 min at 37°C. Peptide extraction was repeated and the supernatant pooled with the earlier supernatant (124). The final pooled supernatant was vacuum centrifuged to 3 µl.

2.6 Mass Spectrometry

2.6.1 MALDI-TOF

MALDI-TOF was used for the peptide mass fingerprint analysis (PMF) of protein spots identified in chapter 3. 0.5 µl of the peptide digest sample to be analysed was spotted onto the MALDI target plate. The remainder was concentrated using a Zip-Tip C18 (Millipore, Billerica, MA, USA) and also spotted onto the target plate. 0.5 µl of α-cyano-4-hydroxycinnamic acid solution was layered over the sample spot. Sample spots were allowed to air dry before insertion into the MALDI-TOF.

The MALDI-TOF spectra of the peptides were obtained with an Axima CFR plus mass spectrometer (Kratos analytical, Shimadzu, Manchester UK) in 2 GHz reflectron mode. Near spot calibration was performed using a 5 point calibration mix: Bradykinin [1-5] (572.7 Da), Angiotensin II (1046.2 Da), Neurotensin (1672.9 Da), ACTH [18-39] (2465.7 Da) and Bovine Insulin chain B (3495.9 Da) (Laserbiolabs, France). Acquisition and data processing were controlled by Launchpad software V. 2.4 (Kratos Analytical, Shimadzu, Manchester UK). No smoothing was applied to the spectra. The positive control used was BSA

solution and was subject to the same digestion procedure. The negative control used was a blank gel piece sourced from the same gel under analysis. Peaks from the negative control along with common contaminants listed on the Matrix Science website were eliminated from the raw peak list to create the final peak list for searching with Mascot.

2.6.2 LC-MS/MS

Digestion was carried out as described for MALDI-TOF. Samples were mixed with 5% formic acid in 50% ACN. The separation and analysis of digested peptides were performed using reverse phase (RP) capillary liquid chromatography (Dionex ultimate 3000, Dionex, Sunnyvale, CA, USA) directly coupled to a Finnigan LCQ IT mass spectrometer (Thermofisher, Waltham, MA, USA). A C18 PepMap100 solid-phase extraction m-Precolumn cartridge (particle size 5 mm, pore size 100 Å, 300 mm inner diameter - Dionex) trapping column and Nano-column PepMap C18 reversed-phase material (particle size 3 mm, pore size 100Å, 75 mm inner diameter, Dionex) resolving column were placed in-line. Peptides were bound and preconcentrated in the trapping column using the mobile phase (composition: 0.1% formic acid in 2.5% ACN). The eluting gradient was 2–90% v/v ACN in 0.1% v/v formic acid for 50 min at a flow rate of 0.3 µl/min. Eluent from the capillary column was directly sprayed into the ion trap mass spectrometer.

All the data were collected in centroid mode using "triple play" settings (i.e. a full mass scan at mass range of 400– 1500 Da (*m/z*), determination of the charge states of an ion on zoom scan, and then acquisition of the MS/MS spectrum of each ion on a full MS/MS scan, with collision energy preset at a value of 55%). Calibration of the mass spectrometer was carried before each batch of samples using human glufibrinopeptide B.

2.6.3 Mascot® Search

All searches were carried out using in-house Mascot software (Matrix Science, London, UK, at http://www.matrixscience.com). Criteria selected in the search field for PMF analysis included: (1) Database selection - here the PMF obtained for each protein digest was analysed using the Swiss Prot (High quality, curated protein database) and MSDB (Comprehensive, non-identical protein database) non-redundant protein databases against all species, all mammals and all bacteria, with focused analysis on *Homo sapiens* and *Streptococcus pneumoniae*. (2) Missed cleavages was varied between 0 - 2. (3) Fixed post translational modifications (PTM) of carbamidomethyl on the carboxy terminal and variable PTM of oxidation of methionine, phosphorylation, acetylation and propionamide. (4) The error window on experimental peptide mass values (peptide tolerance) was varied between 1 – 1.2 Da.

Criteria for the positive identification of the proteins with MALDI were: (1) Scores above the Mowse statistical threshold value in Mascot using the Swiss Prot and MSDB database. (2) Protein must be identified in at least two of the three replicates by a minimum of five peptides in MALDI-TOF. (3) Protein sequence must have a minimum of 10% sequence coverage.

From *raw* LC-MS/MS files, MS/MS spectra were exported as *dta* (text format) files using LCQ_DTA *raw* file converter software (Matrix science) using the standard settings. *Dta* files were merged into a single Mascot generic format (*mgf*) file. Criteria selected in the search field for Mascot MS/MS ion search analysis included: (1) Database selection - here the MS/MS ion spectra obtained for each protein digest was analysed using the Swiss Prot (High quality, curated protein database) and MSDB (Comprehensive, non-identical protein database) non-

redundant protein databases against all species, all mammals and all bacteria, with focused analysis on *Homo sapiens* and *Streptococcus pneumoniae*. (2) Missed cleavages was varied between 0 – 2. (3) Fixed post translational modifications (PTM) of carbamidomethyl on the carboxy terminal and variable PTM of oxidation of methionine, phosphorylation, acetylation and propionamide. (4) The error window on experimental peptide mass values (peptide tolerance) was varied between 1 – 1.2 Da. (5) The Error window for MS/MS fragment ion mass values (MS/MS tolerance) was set to 0.6 and (6) the precursor peptide charge state was varied between +2 and +3.

2.6.4 Mascot® Statistics

Criteria for positive identification of the proteins with LC-MS/MS: (1) Scores above 100 according to the Mowse statistical threshold value in Mascot using the Swiss Prot database were considered significant. (2) Protein must be identified in at least two of the three replicates, by a minimum of two peptides (unless previous identification with MALDI TOF). The threshold ions scores suggested by Mascot for confident PMF identification in Swiss Prot and MSDB databases were 55 and 63 ($p < 0.05$), respectively. The threshold score for MS/MS data was 31 for individual peptides (120).

2.7 Western Blot

2.7.1 Preparation of CSF Samples for Western Blot

CSF samples were measured for protein concentration in order to determine an optimum quantity of protein. A volume (µl) equivalent to the required weight of protein in CSF was combined with PBS to make a final volume of 10 µl. To this volume 2x sample buffer was added to make up a final volume of 20 µl. The samples were boiled for 3 min at 95°C to denature the samples before SDS PAGE.

2.7.2 Western blot of CSF

Solutions for Western blotting were made up on the day of the experiment.

2.7.3 Protein Transfer

Transfer of SDS PAGE separated protein to membrane was undertaken using the Towbin method. Here the protein is transferred using electric current onto a membrane in Towbin transfer buffer at 4°C. The membranes used in all the experiments of this analysis were nitrocellulose paper (NCP, GE lifesciences). AIF and caspase 3 protein blots were retested using PVDF membranes (GE Lifesciences)

Depending on the antibody to be used for investigation, the membranes were blocked using 5% milk or 5% BSA in TBS-T. Blocking was carried out overnight at 4°C on a rocking platform. The molecular weight markers were coloured markers which can transfer onto the membrane (Full-Range Rainbow Molecular Weight Markers (GE Lifesciences RPN800E). The protein markers consisted of: 225 kDa (Blue), 150 kDa (Red), 102 kDa (Green), 76 kDa (Yellow), 52 kDa (Purple), 38 kDa (Blue), 31 kDa (Orange), 24 kDa (Green), 17 kDa (Blue) and 12 kDa (Red).

2.7.4 Antibody Detection

Membranes were then incubated in primary antibody for 2 hrs at RT on a rotating platform or overnight at 4°C on a rocking platform. Primary antibody was added to either 5% milk or 5% BSA in TBS-T (depending on the primary antibody). Membranes were then washed 3 times for 20 min at RT to remove primary antibody before the addition of the secondary antibody in either 5% milk or 5% BSA in TBS-T for 2 hrs at RT on a rotating platform. The secondary antibody was then washed 3 times for 20 min at RT.

Depending on the conjugate attached to the secondary antibody used the membrane was then either incubated in DAB substrate, BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/ nitro blue tetrazolium, Sigma Aldrich) or ECL (Enhanced Chemiluminescence, GE lifesciences). Both DAB and BCIP/NBT produce precipitated coloured blots directly onto the membrane surface. ECL emits chemiluminescence which is detected using x-ray film (Kodak). The x-ray film was then developed using an automated x-ray developer (Photon Imaging, Swindon, UK).

2.7.5 Semiquantitative Measure of Protein Expression

The protein images were captured using a gel documentation camera (Syngene, Cambridge, UK). The images were converted to TIFF format before analysis using the open source software ImageJ (V. 1.43, NIH, Maryland, USA, free download available at http://rsb.info.nih.gov/ij/) (125). Here the images were initially converted to an 8-bit format to covert the image to true greyscale. The background was subtracted from the image before inverting the images to black and grey. The bands of interest after inverting the colours appear as dense grey bands. The software then measures the absolute intensity of each band using pixelated grey density. This can be converted to a relative intensity ratio using a standard band as reference. In these Western blots the reference band used was the positive control for the protein under analysis. The relative intensity is a unitless value. By calculating a relative intensity that is tied to the same sample standard on every membrane, we can make up for variations in the length of film exposure or variations in the efficiency of the antibodies or other reagents. This can be converted to an approximate quantity using a calibration curve generated from the positive control as shown in figure 2.1. This value was then converted to a native concentration in CSF using the total preparation volume

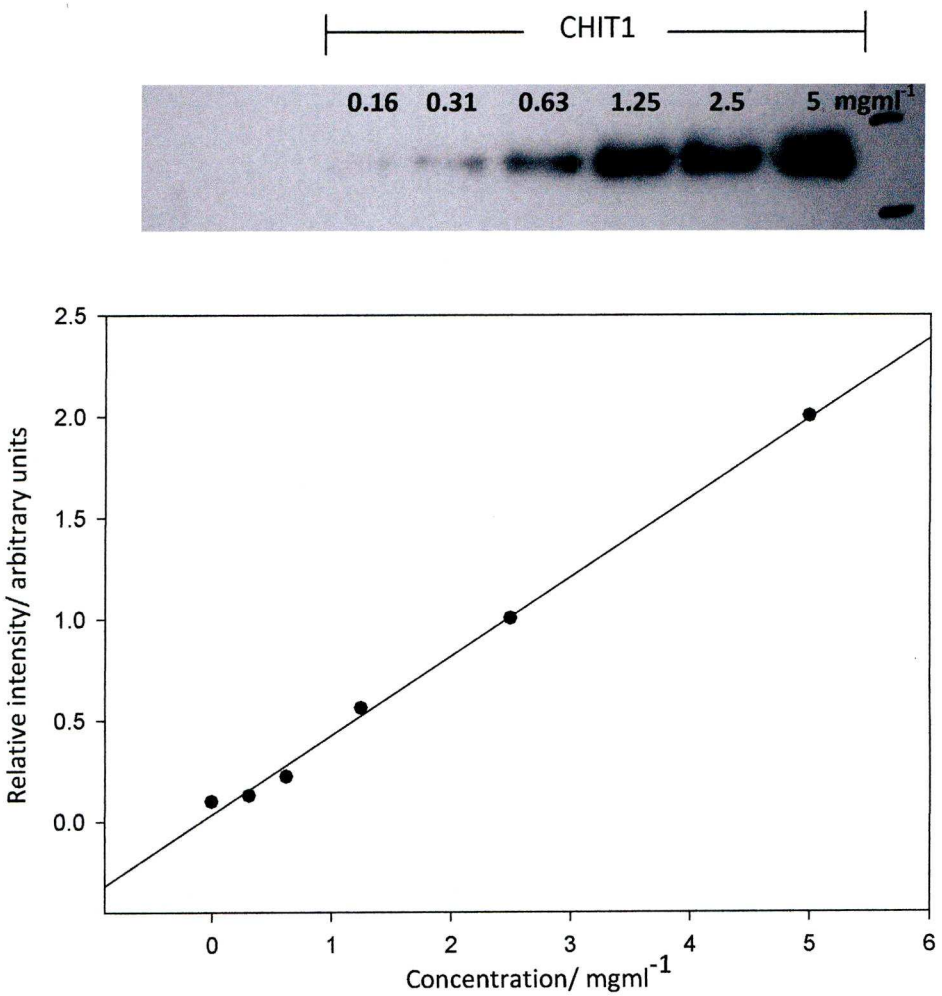

Figure 2.1 Quantification of Western Blot Expression. *Each of the protein controls was serially diluted. Using ImageJ the relative intensity of the protein band was matched against the quantity of protein loaded onto the gel (R^2 = 0.99). This was used to determine approximate values for the quantity of protein expressed.*

multiplied by the total protein concentration expressed as a ratio of volume of protein loaded onto the gel.

The protein images used in chapter 6 were captured with a GS-710 Imaging Densitometer (BioRad, Hemel Hempstead, Hertfordshire, UK). The images were saved in TIFF file format before semiquantitative analysis using PDQuest® software v. 6.2.1 (BioRad).

CHAPTER 3

2D PAGE OF CSF IN PATIENTS WITH PNEUMOCOCCAL MENINGITIS

3.1 Introduction

Proteomics offers a novel methodology for the analysis of complicated biological samples. It also provides a promising approach to monitor the pathological changes in patients with neurological disorders. The proteome has been described as all of the proteins produced by an organism, cell or tissue under defined conditions.

For pneumococcal meningitis, the CSF proteome could provide unique protein biomarkers for the pathways associated with disease progression or proteins associated with cell death pathways (126). This could lead to novel treatment interventions or improvements in current therapy. Human body fluids, such as CSF and plasma, are rich sources of proteins and in particular glycoproteins, which are recognised diagnostic markers of disease (110;127).

One of the basic requirements for proteome studies is the need for a separation method that is capable of separating very complex protein mixtures, even many thousands of proteins in one experiment. At present, 2D PAGE is the simplest method for the separation of complex protein mixtures in proteomics research. 2D PAGE provides an initial basis for comparing a selection of proteomes for potential diagnosis and instant proteome comparison. The differentially expressed proteins can be located on a 2D gel by a comparison of the proteomes contained in the digitised gel images obtained from the CSF proteomes of controls and patients (128).

2D PAGE was used to test three hypotheses. (1) There is a difference between the 2D gel profile of clinically normal (control) CSF and clinically diagnosed meningitis CSF. (2) There are differences between the 2D gel profile of non-survivors and survivors of

pneumococcal meningitis. (3) The meningitis CSF contains pneumococcal proteins as well as host response proteins.

3.2 Materials and Methods

All chemicals were purchased from Sigma Aldrich (Poole, UK), VWR (Lutterworth, UK) or Fisher (Loughborough, UK) unless otherwise stated. General solutions used for the analysis were made using HPLC grade water. The pH of all solutions used in this chapter was adjusted by the addition of 1 M HCl or NaOH. Other solutions used in this chapter include: (1) Rehydration solution (composition: 8 M urea (Sigma), 3% w/v CHAPS, 0.5% w/v IPG buffer 3–10 NL (GE Lifesciences), 10 mM DTT, and 0.5 mg bromophenol blue. (2) Equilibration buffer (composition: 50 mM Tris-HCl pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS, and 0.5 mg bromophenol blue). (3) Reducing solution (composition: 0.1g DTT and 10 ml equilibration buffer), and (4) Alkylating solution (composition: 0.45 g iodoacetamide in 10 ml of equilibration buffer).

3.2.1 Patient CSF Sample Selection

The 2D PAGE analysis included patient samples from the rapid death group (non-survivors, n = 11) and the well group (survivors, n = 9). 'Normal' CSF was obtained from patients who had presented with symptoms and found to have no evidence of infection on lumbar puncture (n = 10). Details of patients used in this analysis are given in table 3.1.

3.2.2 Sample Preparation

CSF Preparation

CSF samples were stored at -20°C within an hour of sampling and at -80°C from 24 hrs until the time of analysis (See appendix A). Archived

Table 3.1 Clinical details of patients used in pilot proteomics

	Cerebrospinal fluid sample		
	Normal	***Non-survivors***	***Survivors***
	N = 10	N = 9	N = 11
Age - yrs mean (+/- SD)	27.8 (9.5)	28.3 (4.8)	35.0 (14.7)
Male sex - number	3	2	5
Glasgow Coma Score - mean (+/- SD)	9.4 (4.7)	11.6 (2.8)	8.0 (3.0)
Mean time to presentation (Interquartile range)	55.2 (12 - 96)	50 (18 - 72)	86.5 (10 - 168)
Previous antimicrobials - number	2	0	0
Positive CSF culture (%)	-	9 (100)	11 (100)
Positive blood culture (%)	-	3 (33)	4 (36)
Blood culture unavailable	-	1	0
Hemoglobin, g/dL (+/- SD)	-	9.8 (1.4)	10.2 (3.3)
Steroid Treatment – number (placebo)	-	0 (9)	0 (11)
HIV Positive (% of those tested)	-	9 (100)	11 (100)
HIV Not known	10	-	-
Survival at day 10	-	0	11

The table above shows the patient data for CSF samples used in this analysis. All meningitis subjects were diagnosed as having pneumococcal meningitis caused by Streptococcus pneumoniae, all subjects were HIV positive and all samples were collected before any treatment commenced. Normal CSF was obtained from patients who tested negative for meningitis or any other pathogen. The HIV status of the normal patients was not known.

CSF was thawed on ice and desalted using a centrifugal filter (YM3, Amicon and Microcon, Millipore). The salt content in CSF must be removed, or decreased significantly, before 2D PAGE. A high salt content interferes with the electrophoretic separation of proteins because of the high electrical current that is carried by the salt load. This reduces the quality of the 2D PAGE separation.

The concentration of protein was determined using the Bradford assay as discussed in chapter 2. Samples with protein concentrations lower than 1 $mgml^{-1}$ were concentrated by vacuum centrifugation to a volume sufficient for analysis with 2D PAGE.

Pneumococcal Culture Protein Extraction

In order to compare and confirm the presence of pneumococcal proteins in meningitis CSF, protein from pneumococci was extracted and compared against meningitis CSF. The pneumococcal types cultivated were serotype 1, 6b and 14. The bacteria were cultured on human blood-agar plates at 37°C in 5% CO_2 overnight. The pneumococci were then cultivated in Todd-Hewitt media supplemented with yeast extract (TH-Y, BD sciences) overnight or until mid-log phase (O.D. of 0.4 – 0.6 at 620 nm). Bacterial samples were "snap" frozen in liquid nitrogen. A proteome extraction kit (Qiagen, Valencia, CA, USA) was used in accordance with the manufacturer's instructions to extract bacterial proteins via the freeze thaw cycle. Protein was separated from the TH-Y media by ice cold acetone protein precipitation. Protein content was measured using the Bradford assay as described. The protein yielded from this was analysed using 2D PAGE with the same conditions as used for CSF samples.

3.2.3 2D PAGE

For each CSF sample a volume equivalent to 75 µg (for silver staining) of total protein was brought to a final volume of 260 µl with

rehydration solution. This solution was added to a dry strip re-swelling tray. IPG strips (13cm, 3–10 non linear, GE Lifesciences) were placed into the lanes of the re-swelling tray with the protein/rehydration buffer mix. In-gel rehydration was carried out for 20 hrs at room temperature (129).

After rehydration, isoelectric focussing was carried out using a Multiphor™ II flat bed electrophoresis system (Pharmacia, Uppsala Sweden). Proteins were focused at 300 V (1 hr), 1500 V (1 hr), and then 3000 V (20 hrs). The IPG strips were equilibrated at room temperature in reducing solution and then alkylating solution for 15 min each.

After equilibration, the IPG strips were applied to vertical 12.5% SDS polyacrylamide gels (gel plate size 180 x 160 x 1.5 mm). Electrophoresis was performed at room temperature using a constant current of 25 mA *per* gel using an SE 600 series vertical slab gel electrophoresis units (Hoefer, Little Chalfont, Buckinghamshire, UK). For each sample, 2D PAGE was repeated in duplicate. The standard molecular weight marker proteins consisted of hen egg white conalbumin (76 kDa), bovine serum albumin (66.2 kDa), Bovine muscle actin (43 kDa), rabbit muscle GAPDH (36 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and equine myoglobin (17.5 kDa).

2D gels were stained with silver stain because of its high detection sensitivity (130;131). The smallest protein spot visible with silver staining generally correlates to one nanogram of protein. That amount (*ca.* hundreds of fmol, depending on each protein's *Mr*) generally suffices for the mass spectrometric characterisation of the proteins (see chapter 2) depending on the sensitivity of the mass spectrometry method.

3.2.4 2D PAGE Comparison Software

The gels under analysis were silver stained in order to detect low abundance proteins. The abundance of protein spots on the 2D PAGE gels was analysed by scanning the spots with a PowerLook 111 scanner (UMAX, Taiwan). The gels were compared using Progenesis software PG220 (Nonlinear Dynamics, Newcastle, UK). The gels were analysed in four groups; normal (control 1), bacteria (control 2), non-survivors and survivors. The order of analysis included:

Reference Selection

A reference gel was selected which encompassed the greatest number of spots. In addition a gel was selected to represent each group to produce an 'averaged reference' gel for each of the four groups.

Spot Filtering

The spots generated were manually filtered to remove erroneous spots which would have confounded the final result. This was reduced as much as possible in order to avoid potential adverse effects on the outcome of the analysis. The background was subtracted using the 'Mode of none spot' method which calculates the background based on the most common pixel value in the region around the spot.

Warping and Matching

Warping was performed via vector alignment. Manual warping was required for some spots. However this was reduced as much as possible. Manual warping was used to overcome 2D gel swirling, generated in the gel during the isoelectric focusing stage of protein separation. In order to improve matching between the gel spots of the individual gel and the reference gels, a reduced stringency of matching was used. Spots were accepted if present in at least 50% of the gels in each group.

Normalisation

In this analysis normalised volume was used, which is a computerised construct which creates a 3D visualisation of the protein spot based on the intensity of the spot as shown in Figure 3.1. All spots were normalised using the Total spot normalisation method. In this normalisation method, each spot on a gel image is expressed relative to the total volume of all spots on that image. Since this tends to produce extremely small values, the ratio can be multiplied by a user defined scaling factor (in this experiment 100). The software performs a t-test on the normalised volumes. Initially the reference gels are compared within the individual groups to their respective reference gel. Next the averaged gels are compared by comparing the averaged gels with the reference gel.

The 'Total spot normalisation' method is the most appropriate method in situations where images within a single experiment have widely differing numbers of spots, ensuring that all spots will only be normalised relative to those spots that are present on all gels within a group. This method assumes that few protein spots are changing within the experiment, or if they are, that the bulk of changes are averaged out. It also assumes that those spots which are changing don't contribute to a large proportion of the total volume of spots (132).

Progenesis Statistics

In the Progenesis software, the Welch t-test for unequal variance data was used to identify those spots in the two averaged gels that differed in normalised volume for a spot. The null hypothesis being that there was no change in the protein's normalised volume between the groups.

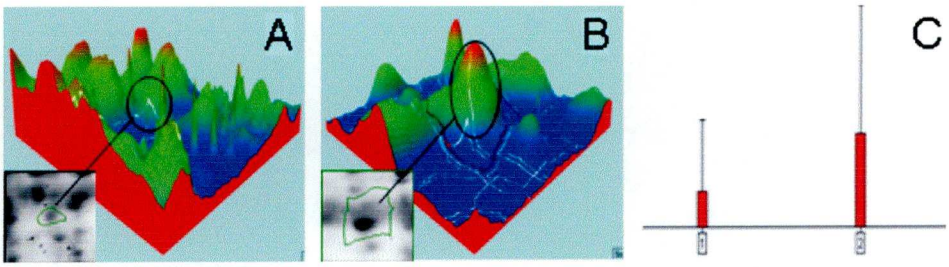

Figure 3.1 Comparison of Normalised volume. *To accurately compare the abundance of protein spots across 2D gels, it was essential to compensate for variations from external sources of interference caused by such things as variations in staining time. Normalisation is the process of removing such variation. In this analysis normalised volume was used. This is a computerised construct which creates a 3D visualisation of the protein spot based on the intensity of the spot. For example when two spots are matched. In gel* ***A****, the spot was found to have a low normalised volume compared to the same spot in gel* ***B****. This normalised volume can be compared between gels in order to determine if there is a difference in intensity (represented in the bar chart,* ***C****).*

3.3 Results

3.3.1 Cerebrospinal Fluid Sample Information

In the initial experiment, the 2D gel profile of 30 CSF samples was compared. Analysis was carried out on clinically normal CSF (n = 10) and clinically diagnosed pneumococcal meningitis CSF (n = 20). These 20 meningitis CSF samples were used as representative of the spectrum of protein profiles in meningitis CSF. It was observed that protein saturation occurred in the 2D gel analysis beyond this number of samples. Saturation occurred as a result of protein spot themes developing amongst all the gels and also protein spot metathemes developing i.e. themes within the groups. This saturation effect was a result of basic spot patterns caused by endogenous proteins within CSF.

In addition these samples had the largest distribution of concentrations of proteins found in the collection of CSF. Therefore the proteins which were present in these 20 samples were most likely to represent the proteomic profile of the CSF collection as a whole. All subjects were diagnosed as having pneumococcal meningitis caused by *Streptococcus pneumoniae*, all subjects were HIV positive and all samples were collected before any treatment commenced. Normal CSF was obtained from patients who tested negative for meningitis or any other pathogen.

3.3.2 CSF Protein Concentration

The concentration of protein in each sample was measured using the Bradford assay. The 'normal' samples had a protein concentration ranging from 0.05 – 0.94 $mgml^{-1}$ (average 0.23 $mgml^{-1}$). The non-survivors had a protein concentration of 2.33 – 10.40 $mgml^{-1}$ (average 6.94 $mgml^{-1}$). The survivors had a range of protein concentration of 3.16 – 10.89 $mgml^{-1}$ (average 7.07 $mgml^{-1}$, Figure 3.2). The protein

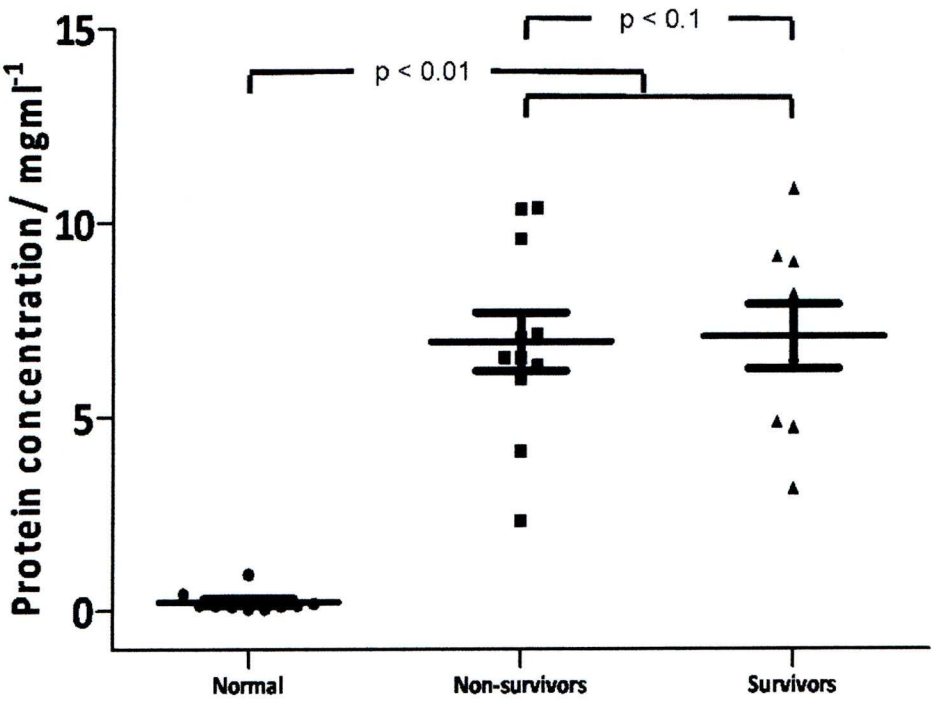

Figure 3.2 Protein concentrations observed in initial CSF samples CSF. *The total protein concentration of CSF did vary between survivors and non-survivors ($p < 0.1$). However both non-survivor and survivor CSF had a significantly greater concentration of protein compared to normal CSF ($p < 0.01$).*

concentration in normal CSF was significantly less than clinically diagnosed meningitis CSF ($p < 0.01$).

3.3.3 Progenesis Analysis:

Differences between CSF 2D Gel Profiles

For this experiment the gels were analysed in three groups; normal (control), non-survivors and survivors (Figure 3.3). Initially the normal CSF gels were compared against the clinically diagnosed meningitis CSF gels. Normal CSF was found to have an average of 638 protein spots per gel. The average number of protein spots identified in clinically diagnosed meningitis CSF gels was 2466. There were 20 protein spots in normal CSF which were common to clinically diagnosed meningitis CSF gel spots as shown in figure 3.4A. Therefore there were over 2400 proteins which were novel to meningitis CSF. All of these 20 spots matched were at least five-fold lower in expressed normalised volume in the normal CSF samples compared to the clinically diagnosed meningitis CSF. These data are shown in table 3.2.

The average number of protein spots identified in the non-survivor gels was 2386 and the survivor gels had an average of 2546 protein spots per gel.

The normalised volume data for all spots on the average non-survivor gel was compared with the normalised volume data for all spots on the averaged survivor gel. There were 469 common spots between non-survivors and survivors (present in a minimum of 50% of gels). This comparison identified 34 proteins with a minimum 2-fold difference in normalised volume between non-survivors and survivors as summarised in table 3.3. All of these proteins identified had a significantly lower concentration in the normal CSF compared to the clinically diagnosed meningitis CSF. None of the 20 protein spots which were common to

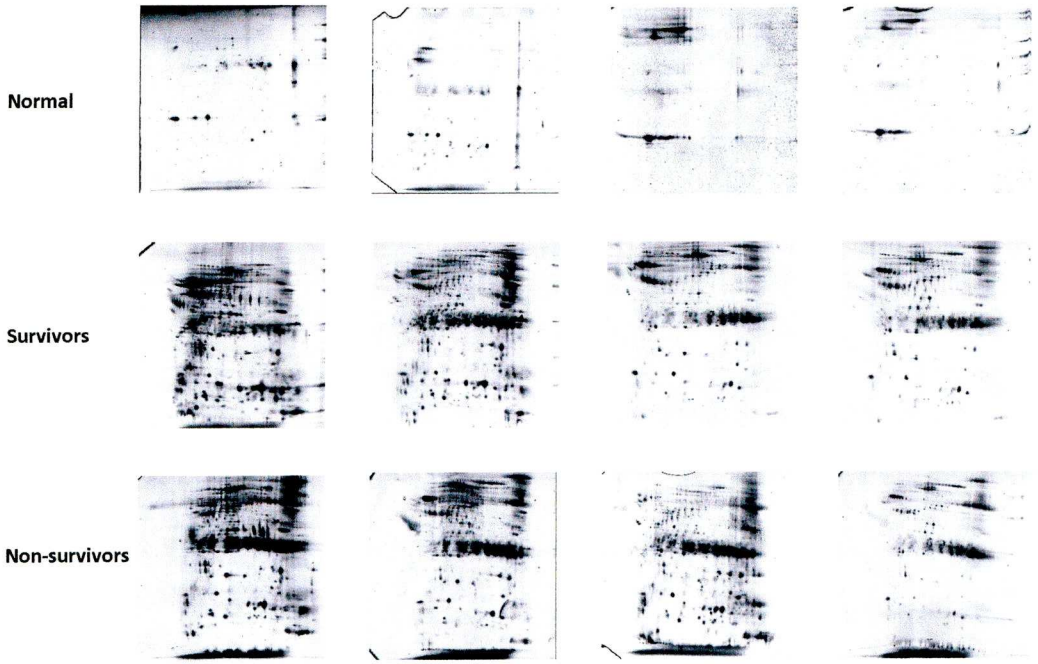

Figure 3.3 Representative CSF 2D gels. *In this figure four gels were selected as representative of the protein profiles observed in the 2D gels. The most concentrated 2D gel is shown on the left with the least concentrated on the right. CSF was separated using 2D PAGE. The gels were grouped into three groups of normal's, survivors and non-survivors. 2D PAGE separates proteins based on isoelectric point and molecular weight. The proteins are visualized using silver stain and analysed for differences. It can be observed that the normal CSF has less protein compared to the survivors and non-survivors. It can also be observed that both groups show a wide range of appearances. (The entire collection of gels is shown in appendix C).*

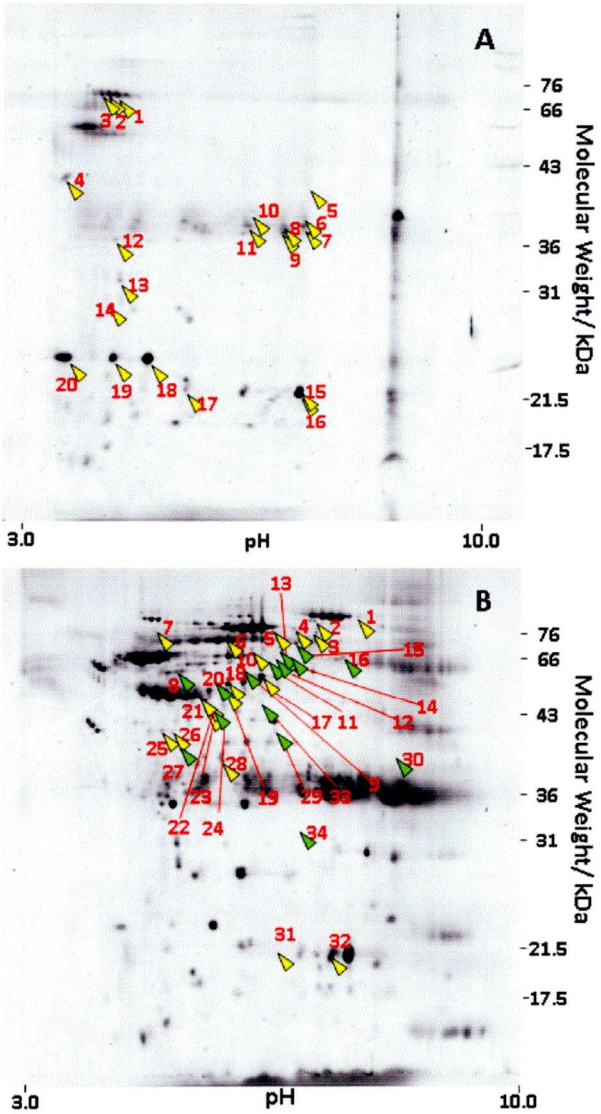

Figure 3.4 Comparison of 2D gels of normal and meningitis CSF. *The green arrows represent upregulated protein spots and the yellow arrows represent downregulated protein spots. Spots were accepted provided they were present in a minimum of 50% of the gels analysed in that group. In* ***A*** *the normalised volume of each spot identified in normal CSF was compared to the normalised volume of each spot identified in diseased CSF. There were 20 protein spots found to be common between diseased and normal CSF. All 20 proteins were significantly upregulated in normal CSF. In* ***B*** *there were 34 differential expressed proteins found between survivors and non-survivors of meningitis.*

Table 3.2 Spots in Normal CSF compared to Diseased CSF

Spot no.	Experimental Mr/pI (kDa/pH)	Normal Median	95% CI	Diseased Median	95% CI	Expression Fold difference in Normal CSF
1	66, 4.50	0.003	0.001 – 0.006	0.017	0.009 – 0.036	-6.0
2	66, 4.50	0.002	0.002 – 0.090	0.016	0.010 – 0.056	-9.2
3	66, 4.50	0.002	0.000 – 0.012	0.018	0.020 – 0.089	-7.5
4	40, 4.00	0.002	0.000 – 0.034	0.033	0.027 – 0.098	-16.4
5	40, 7.00	0.001	0.001 – 0.078	0.005	0.018 – 0.078	-5.5
6	38, 7.00	0.004	0.001 – 0.034	0.038	0.042 – 0.120	-10.7
7	36, 7.00	0.004	0.007 – 0.013	0.030	0.026 – 0.110	-8.1
8	35, 6.90	0.003	0.004 – 0.016	0.037	0.094 – 0.106	-11.0
9	35, 6.90	0.003	0.000 – 0.085	0.034	0.025 – 0.078	-9.9
10	36, 6.00	0.002	0.002 – 0.013	0.042	0.050 – 0.160	-23.6
11	36, 6.00	0.006	0.001 – 0.023	0.032	0.026 – 0.098	-5.0
12	31, 5.00	0.003	0.000 – 0.078	0.017	0.023 – 0.089	-5.4
13	27, 5.10	0.003	0.000 – 0.010	0.056	0.050 – 0.090	-21.3
14	21, 5.00	0.003	0.000 – 0.034	0.018	0.048 – 0.078	-6.4
15	19, 7.00	0.003	0.001 – 0.067	0.034	0.022 – 0.098	-10.4
16	19, 7.00	0.001	0.000 – 0.012	0.005	0.018 – 0.078	-5.5
17	20, 5.50	0.002	0.000 – 0.067	0.031	0.108 – 0.230	-16.7
18	22, 5.40	0.004	0.000 – 0.034	0.037	0.094 – 0.120	-9.1
19	22, 5.00	0.004	0.001 – 0.016	0.028	0.065 – 0.130	-7.1
20	22, 4.50	0.001	0.000 – 0.018	0.010	0.046 – 0.120	-14.7

The 20 spots found to be common between diseased and normal CSF is shown above. The median of the data and lower to upper 95% confidence interval (CI) are shown along with the expression value as fold difference in normal CSF compared to CSF from patients clinically diagnosed with meningitis.

Table 3.3 Spots in non-survivors compared to survivors

Spot no.	Experimental Mr/pI (kDa/pH)	Non-survivors Median	95% CI	Survivors Median	95% CI	Expression Fold Difference in Non-survivors
1	76, 6.30	0.012	0.005 - 0.020	0.059	0.002 - 0.117	-4.8
2	66, 5.90	0.008	0.002 - 0.014	0.022	0.006 - 0.038	-2.8
3	60, 5.90	0.012	0.005 - 0.019	0.047	0.010 - 0.085	-4.9
4	60, 5.80	0.019	0.006 - 0.032	0.083	0.000 - 0.203	-5.0
5	59, 5.60	0.013	0.007 - 0.018	0.052	0.000 - 0.113	-3.6
6	58, 5.30	0.027	0.006 - 0.048	0.070	0.028 - 0.112	-2.5
7	60, 4.70	0.010	0.000 - 0.024	0.021	0.005 - 0.037	-3.0
8	58, 4.90	0.050	0.002 - 0.098	0.025	0.000 - 0.051	2.9
9	48, 5.81	0.130	0.047 - 0.213	0.032	0.000 - 0.066	5.1
10	50, 5.50	0.025	0.015 - 0.035	0.057	0.023 - 0.091	-2.5
11	50, 5.60	0.118	0.038 - 0.197	0.035	0.005 - 0.066	3.3
12	50, 5.60	0.068	0.030 - 0.106	0.026	0.011 - 0.042	2.5
13	50, 5.60	0.027	0.000 - 0.053	0.009	0.004 - 0.014	2.5
14	60, 6.00	0.086	0.010 - 0.161	0.029	0.001 - 0.057	3.6
15	60, 6.00	0.050	0.016 - 0.084	0.018	0.000 - 0.039	2.7
16	50, 6.20	0.053	0.023 - 0.083	0.033	0.017 - 0.049	2.0
17	43, 5.60	0.075	0.028 - 0.122	0.179	0.094 - 0.264	-2.0
18	43, 5.40	0.045	0.004 - 0.086	0.104	0.071 - 0.138	2.7
19	40, 5.40	0.085	0.056 - 0.115	0.195	0.121 - 0.269	-2.2
20	55, 5.20	0.095	0.029 - 0.162	0.039	0.022 - 0.056	3.0
21	40, 5.00	0.027	0.005 - 0.050	0.117	0.057 - 0.178	-5.5
22	40, 4.70	0.055	0.000 - 0.118	0.097	0.000 - 0.210	-2.1
23	40, 4.70	0.032	0.001 - 0.055	0.061	0.029 - 0.093	-2.5
24	40, 5.20	0.083	0.030 - 0.135	0.023	0.009 - 0.038	3.4
25	38, 5.20	0.026	0.009 - 0.044	0.051	0.025 - 0.077	-2.2
26	36, 4.75	0.013	0.005 - 0.021	0.038	0.014 - 0.062	-2.5
27	38, 5.10	0.022	0.006 - 0.037	0.007	0.000 - 0.014	3.1
28	35, 5.40	0.022	0.008 - 0.036	0.061	0.010 - 0.112	-2.9
29	40, 5.70	0.063	0.000 - 0.129	0.035	0.014 - 0.055	2.1
30	37, 7.10	0.022	0.000 - 0.044	0.007	0.002 - 0.013	2.8
31	20, 5.60	0.005	0.000 - 0.010	0.014	0.001 - 0.027	-3.8
32	20, 6.00	0.169	0.000 - 0.346	0.392	0.130 - 0.654	-2.0
33	50, 5.90	0.053	0.022 - 0.083	0.023	0.016 - 0.030	2.7
34	27, 5.90	0.027	0.000 - 0.056	0.007	0.001 - 0.013	4.0

The 34 spots found to differ between survivors and non-survivors of meningitis are shown above. The median of the data and lower to upper 95% confidence interval (CI) are shown along with the expression value as fold difference in non-survivors compared to CSF from survivors.

clinically diagnosed meningitis CSF and normal CSF was matched to any of the protein spots in the list of spot differences found between non-survivors and survivors.

Pneumococcal Protein Spots in Clinically Diagnosed Meningitis CSF

In this experiment protein was extracted from cultured pneumococci. The protein yield from the pneumococcal protein extraction was relatively low and the subsequent expression of protein spots was weak. The concentration of protein in sp1 = 0.23 $mgml^{-1}$, sp6b = 0.19 $mgml^{-1}$ and sp14 = 0.14 $mgml^{-1}$. The gels were analysed as described previously. The number of protein spots identified in SP1 was 815, sp6b was 789 and sp14 was 737. These gels were used to produce an averaged pneumococcal gel which had 350 common protein spots between the gels. Of these 350 proteins only three proteins were matched to proteins in clinically diagnosed meningitis CSF gels as shown in figure 3.5.

3.4 Discussion

3.4.1 Normal CSF Protein Differences

In the initial analysis normal CSF was compared to clinically diagnosed meningitis CSF. The concentration of protein in normal CSF was significantly lower compared to clinically diagnosed meningitis CSF. This was visually striking from the 2D gel images. There was a clear difference between the protein profile in normal CSF gels and the protein profile in clinically diagnosed meningitis CSF gels.

Over 2400 proteins were unique and present only in the meningitis CSF. Based on our knowledge of the pathology of this condition it is highly likely that these proteins are present in CSF as a direct consequence of meningitis. There are a number of explanations that could explain the observation; they could potentially be a result of the

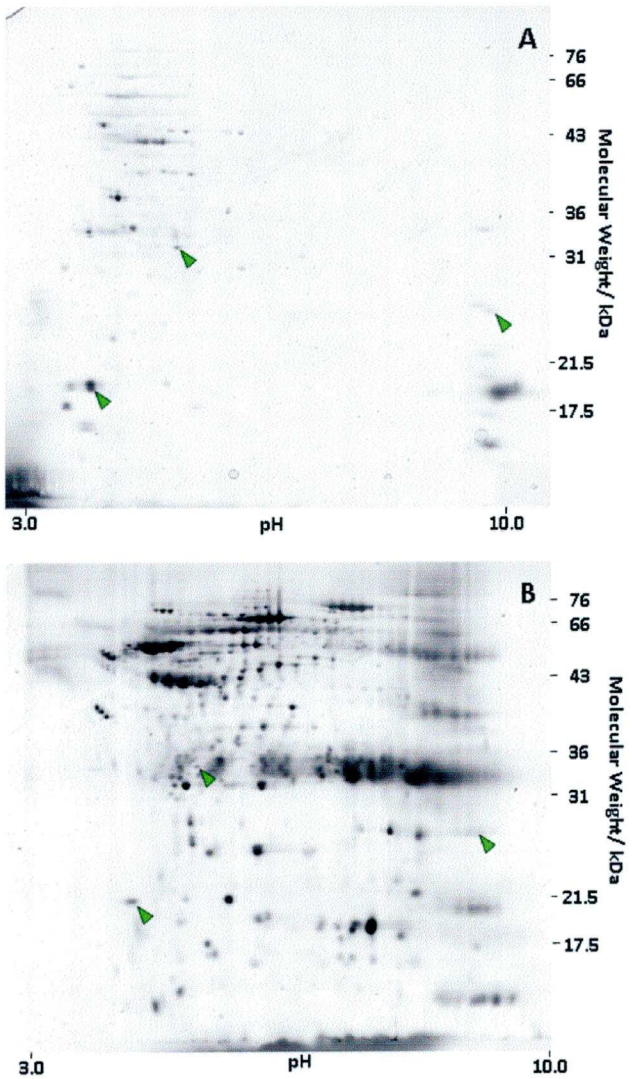

Figure 3.5 Comparison of pneumococcal protein lysate with clinically diagnosed meningitis CSF. *Pneumococcal protein was extracted from cultured pneumococci. This cultured pneumococcal protein was separated using 2D PAGE. The subsequent 2D gel profile was compared to clinically diagnosed meningitis CSF.* ***A*** *- An averaged pneumococcal protein gel was created using the pneumococcal types, sp1, sp6b and sp14.* ***B*** *- There were three protein spots found to be common in pneumococcal protein gels and clinically diagnosed meningitis CSF gels.*

breakdown of the blood-brain barrier: a consequence of the pneumococcal response: due to a significant upregulation of protein within CSF itself (host response) or some combination of the above. There are many proteins which have been discovered to be associated with meningitis. It is likely that these proteins spots will include host response proteins including cytokines, chemokines and growth factors (133). In addition it is likely that pneumococcal proteins will also be amongst these proteins.

3.4.2 Non-survivors Compared to Survivors

In the second analysis the non-survivor CSF average gel was compared to the survivor CSF average gel. A number of the samples had protein spots which were common to all the gels. These endogenous proteins demonstrated that the 2D gels were reproducible. However there were a large number of spot differences within individual gels of both the non-survivor and survivor groups. Also there were a large number of protein spots that were unique to individual samples. These were excluded from the analysis. There were common proteins between non-survivors and survivors which differed in their total expression.

3.4.3 Pneumococcal Proteins in Clinically Diagnosed Meningitis CSF

In the third analysis pneumococcal proteins were compared against meningitis CSF proteins. The number of proteins identified from the pneumococcal protein extraction were similar to spot counts obtained from other research groups as demonstrated in Encheva et al (134). The yield of protein extracted from the pneumococcal culture was low. This was most likely a result of the extraction procedure which was optimised to avoid inhibiting the 2D PAGE procedure.

In this analysis only three protein spots were common between the pneumococcal protein 2D gel average and the meningitis CSF 2D gel

average. This would indicate that the proteins in meningitis CSF are composed predominantly of host proteins with a very small number of pneumococcal proteins.

Although the pneumococcal proteins were useful in establishing what proteins were present in the meningitis CSF gel i.e. either host response proteins or pneumococcal proteins they were not the ideal solution. A more accurate control would have involved culturing pneumococci in an artificial CSF and comparing the proteins produced. However based on the complexities seen the amount of endogenous proteins may have confounded the identification of pneumococcal proteins.

3.4.4 Strengths and Weaknesses of Using 2D PAGE

The technique of separating proteins on a global scale using 2D PAGE is a complex and labour intensive process despite rapid advances in the technology associated with this technique. Also the complexity of Progenesis software was a rate-limiting factor in the analysis. 2D PAGE, however, has been successfully used to analyse cerebrospinal fluid for other diseases such as Alzheimer's disease and Creutzfeldt-Jakob disease (135;136). It has been found to be useful in identifying biomarkers in a number of diseases, such as cancer and has lead to the development of drugs used in the treatment of that disease (137). It was for this reason it was considered a good strategy for identifying proteins of interest associated with pneumococcal meningitis.

2D PAGE allowed for a substantial number of CSF protein spots to be separated and observed. The sensitivity of silver staining allowed many thousands of proteins to be visualised in the CSF proteome. In our hands the 2D gels were highly reproducible despite other papers suggesting otherwise. The process of normalisation in Progenesis tended

to negate the effect of differences from staining or co-migration of spots. However the analysis has suggested some interesting areas where potential biomarkers of interest might be found for further analysis.

3.5 Conclusions

In this chapter it has been shown that there is a significant difference in the protein profile of normal CSF and meningitis CSF. There are also a number of differences between non-survivors and survivors. However the analysis is complicated by the fact that there are a number of protein spot differences within the individual groups also.

It has been demonstrated that meningitis CSF is composed of both host response proteins and pneumococcal proteins. However the pneumococcal proteins are largely confounded by the host response proteins.

The next step for this analysis will require protein spots to be excised out and formally identified. Identification of the excised spot can be achieved by subjecting the protein spots to in-gel tryptic digestion. The trypsin digested fragment can then be analysed by other techniques in particular, liquid chromatography- tandem mass spectrometry (LC-MS/MS) or Matrix assisted laser desorption/ ionisation time of flight (MALDI-TOF) mass spectrometry.

CHAPTER 4

IDENTIFICATION OF PROTEINS IN CSF OF PATIENTS WITH PNEUMOCOCCAL MENINGITIS

4.1 Introduction

Mass spectrometry is a powerful analytical technique which is used to analyse small molecules and complex biomolecules, such as DNA, lipids, carbohydrates and proteins. The scope of the use of mass spectrometry in the biological sciences has grown enormously with applications to all areas concerned with events occurring at the molecular and macromolecular level. One of the most impressive aspects of mass spectrometry is the diversity of the technique itself. It allows an insight into biological systems because these mass specific devices can make measurements at the molecular level. In addition it is a bioanalytical technique with almost unparalleled sensitivity, thus providing an ability to mine substantially deeper into the serum or plasma proteomes than has previously been possible (94).

The technique involves the study of the mass-to-charge ratio (m/z) of atoms, molecules or fragments of molecular ions (112). Spectral information can be obtained that produces only molecular species for molecular mass measurements or fragmentation data of sufficient detail to allow sequencing and structure elucidation.

The mass spectrometry methods used in this chapter include matrix assisted laser/desorption time of flight mass spectrometry (MALDI-TOF-MS) and liquid chromatography electrospray ionisation tandem mass spectrometry (LC-MS/MS). MALDI is more sensitive than other laser ionisation techniques (112). MALDI uses peptide mass fingerprinting of digested proteins to identify proteins whereas LC-MS/MS uses ion fragmentation of digested or intact proteins to identify proteins.

This chapter focuses on the formal identification of the cerebrospinal fluid protein spots selected in chapter 3 as biologically interesting. This

identification has been carried out using modern mass spectrometry and subsequent database searching.

4.2 Materials and Methods

All chemicals were purchased from Sigma Aldrich (Poole, UK), VWR (Lutterworth, UK) or Fisher (Loughborough, UK) unless otherwise stated. General solutions used for the analysis were made using HPLC grade solvents. The pH of all solutions used in this chapter was adjusted by the addition of 1 M HCl or NaOH.

4.2.1 Protein Identification

Sample Selection

Protein spots from 2D gels presented in chapter 3 were selected to be identified using mass spectrometry (Figure 4.1).

4.2.2 Protein Function Clustering

The proteins identified were analysed using three web based protein tools; (1) Protein information resource (PIR, http://pir.georgetown.edu/pirwww/). The data submitted to the PIR was Swiss Prot accession numbers (Appendix D). This tool analyses all the proteins against the Gene Ontology databases.

(2) Gene Ontology (GO, http://www.geneontology.org/). The two main components of the GO that were included in this analysis were 'molecular function' and 'biological process'. Only the GO slim codes are shown (GO slim codes are cut-down versions of the GO ontologies containing a subset of the terms in the whole GO. They give a broad overview of the ontology content without the detail of the specific fine grained terms).

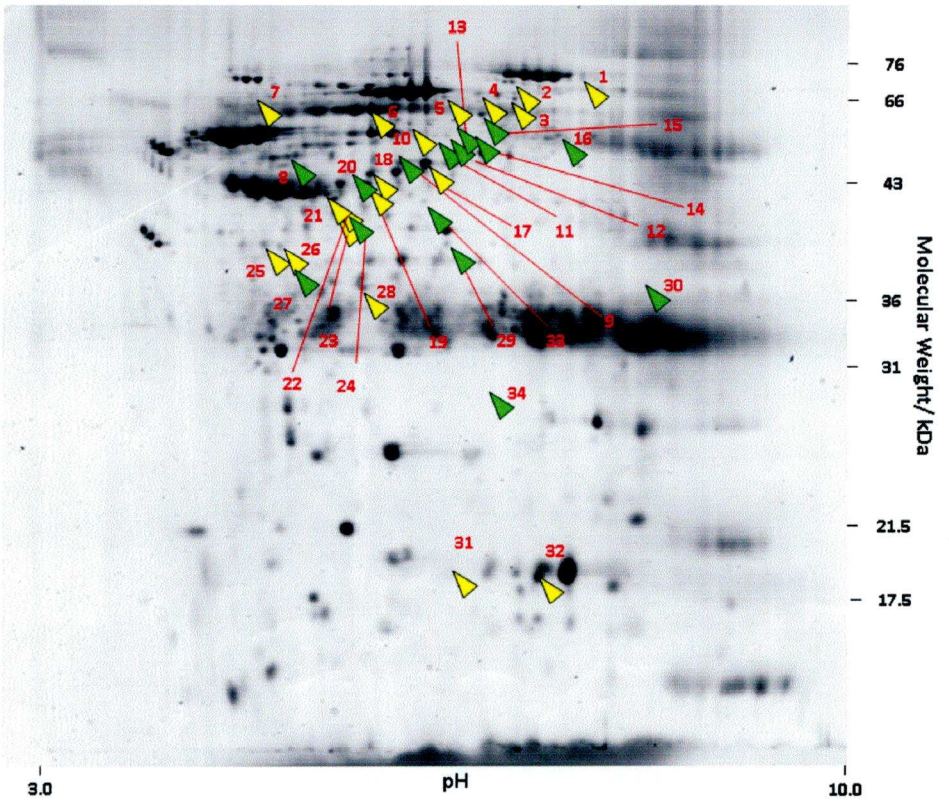

Figure 4.1 Spots selected for identification by mass spectrometry. *The 34 spots found to be differentially expressed between non-survivors and survivors above were excised from the 2D gel using a pen picking method. These spots were then subjected to in-gel tryptic digestion and identification with mass spectrometry.*

(3) ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The protein Pearson (FASTA) sequences generated from mass spectrometry were inputted into ClustalW (122). ClustalW allows the multiple alignments of protein sequences. The basic information they provide is the identification of conserved sequence regions. This is very useful in designing experiments to test and modify the function of specific proteins, in predicting the function and structure of proteins and in identifying new members of protein families. A phylogram was generated (a branching diagram (tree) that is assumed to be an estimate of a phylogeny). The branch lengths were proportional to the amount of inferred evolutionary change.

4.3 Results

Protein spots which were found to be differentially expressed in normal and clinically diagnosed meningitis CSF were excised and analysed with MALDI TOF and LC-MS/MS.

4.3.1 Common Proteins of Normal and Meningitis CSF

The majority of proteins identified in normal CSF which matched to meningitis CSF (n = 20) included immunoglobulin G (IgG, heavy and light chains, 40%) and serum albumin (20%). Other common proteins found included, ceruloplasmin, transthyretin, α-1-antichymotrypsin, apolipoprotein-a-1, apolipoprotein-a-4 and haptoglobin.

4.3.2 Protein Differences between Non-survivors and Survivors

Protein Identification

34 proteins were identified as showing a minimum 2-fold expression difference in non-survivors compared to survivors. The protein spot differences identified are given in full in Table 4.1 with corresponding expression given in appendix E. Protein differences with the largest fold expression included: Chitotriosidase (CHIT1), Serotransferrin (TRFE),

Brain-enriched guanylate kinase-associated (BEGIN), Complement C3 precursor (CO3), phosphoglucomutase-like protein 5 (PGM5), Cleavage stimulation factor 64 kDa subunit tau variant (CSTFT), Beta-2-glycoprotein 1 precursor (APOH), Solute carrier family 25 (member 16, GDC), Fascin (Singed-like protein, FSCN1), complement C1q tumour necrosis factor-related protein 9 (C1QT9), tryptophan/serine protease (YH004), Rab-37 and glyoxalase domain-containing protein 4 (GLOD4).

Functional Clustering

The proteins were cross-referenced using there UniProtKB/ Swiss-Prot accession numbers. The accession numbers were inputted into the PIR database and searched against the GO database. Most of the proteins identified had multiple processes and functions, which made analysis complex (Figure 4.2 and 4.3). Thus by cross referencing the proteins with known aspects of pneumococcal meningitis, the identified protein spots were functionally clustered into the following groups: Membrane and skeletal proteins (n = 4), transporters (n = 2), glycoproteins (n = 2), G proteins of the Ras family (n = 1), metabolic enzymes (n = 4), cellular defence proteins (n = 3), globins (n = 1), kinases (n = 2), phosphatases (n = 3), chaperone (n = 1), protease (n = 1), translation proteins (n = 6), others (n = 3) and proteins of unknown function (n = 1) (Figure 4.4).

Evolutionary Clustering

To determine if there was any evolutionary relationship between the proteins identified a phylogram tree was constructed for the proteins with ClustalW, as shown in figure 4.5. Here we showed that there were 9 groups of close ancestral links between the protein sequences. These links included; group 1 (GLOD4, GDC, PPAL and ZIK1), group 2 (CHIT1, ALBU, and A1AT), group 3 (FSCN1, HNRPC, NLF1, 2AAA, RN112, RXRG, CO3, PRS7, RAB37 and ZA2G), group 4 (FIBB, TRFE, CIA30 and BEGIN), group 5 (CSTFT, APOH and YH004), group 6

Table 4.1 Functional clustering of identified proteins

Protein	*Up or Down regulation*	*Expression in non-survivors*	*References*	*PubMed References*	*Spot*	*Gene Ontology (GO)*		*Category*
						Process	**Function**	
Complement C3 precursor (CO3)	↓	-4.77	(138)	17787	1	0002376: immune system process; 0050789: regulation of biological process; 0050896: response to stimulus; 0051604: protein maturation; 0016044: membrane organization; 0016192: vesicle-mediated transport; 0007154: cell communication	0005515: protein binding; 0030234: enzyme regulator activity	
Chitotriosidase (CHIT1)	↑	5.14	(139)	228	9	0006032: Chitin catabolic process; 0006955: Immune response; 0009617: Response to bacterium	0008061; Chitin binding; 0004568; Chitinase activity	**Cellular defence**
Complement C1q tumor necrosis factor-related protein 9 (C1QT9)	↑	3.42	(140)	65	24	0008152: metabolic process; 0005975: carbohydrate metabolic process; 0050896: response to stimulus; 0051704: multi-organism process; 0002376: immune system process; 0005976: polysaccharide metabolic process; 0009308: amine metabolic process; 0043285: biopolymer catabolic process	0003824: catalytic activity; 0016787: hydrolase activity; 0001871: pattern binding; 0030246: carbohydrate binding; 0043167: ion binding	
T-complex protein 1 subunit zeta (TCPZ)	↓	-2.75	(141)	135	2	0019538 : protein metabolic process; 0006457 : protein folding	0000166 : nucleotide binding; 0001882 : nucleoside binding; 0005515 : protein binding	**Chaperones**
Serotransferrin (TRFE)	↓	-5.47	(142)	19002	21	0006810 : transport; 0006811 : ion transport; 0065008 : regulation of biological quality	0043167 : ion binding; 0005515 : protein binding	**Transporters**
Solute carrier family 25 (member 16) (GDC)	↑	3.97	(143)	38	34	0006810: transport.	0015300: solute antiporter activity.	

Table 4.1 contd.

Protein	*Up or Down regulation*	*Expression in non-survivors*	*References*	*PubMed References*	*Spot*	*Gene Ontology (GO)*		*Category*
						Process	**Function**	
Retinoic acid receptor RXR-gamma (RXRG)	↓	-2.46	(144)	431	10	0006350: transcription; 0050789: regulation of biological process; 0016070: RNA metabolic process; 0032501: multicellular organismal process; 0032502: developmental process	0005496: steroid binding; 0003676: nucleic acid binding; 0004871: signal transducer activity; 0030528: transcription regulator activity; 0043167: ion binding	**Transcription and Translation**
Cleavage stimulation factor (CSTFT) 64 kDa subunit, tau variant (CSTFT)	↑	3.30	(145)	533	11	0006396: RNA processing	0003676: nucleic acid binding; 0000166: nucleotide binding	
Heterogeneous nuclear ribonucleoproteins C1/C2 (HNRPC)	↑	2.46	(146)	1665	12	0006396: RNA processing	0005515: protein binding; 0003676: nucleic acid binding; 0000166: nucleotide binding	
Zinc finger protein 179 (RN112)	↑	2.02	(147)	23	16	0006457: Protein folding.	0016787: hydrolase activity; 0005515: protein binding; 0000166: nucleotide binding; 0043167: ion binding	
Zinc finger protein 1 (ZIK1)	↓	-2.02	(148)	5	17	0006350: transcription; 0050789: regulation of biological process; 0016070: RNA metabolic process	0003676: nucleic acid binding; 0043167: ion binding	
Eukaryotic translation initiation factor 2, subunit 2 beta (EIF2S2)	↓	-2.18	(149)	1513	25	0006412: translation	0003676: nucleic acid binding	
Beta-2-glycoprotein 1 precursor (APOH)	↑	3.61	(150)	1709	14	0050789: regulation of biological process; 0050817: coagulation; 0050878: regulation of body fluid levels; 0050896: response to stimulus; 0065007: biological regulation; 0016265: death; 0032502: developmental process; 0006928: cell motion; 0040011: locomotion; 0006662: glycerol ether metabolic process; 0044255: cellular lipid metabolic process; 0032501: multicellular organismal process; 0008283: cell proliferation	0001871: pattern binding; 0030246: carbohydrate binding; 0043498: cell surface binding; 0008289: lipid binding; 0030234: enzyme regulator activity; 0005515: protein binding	**Glycoproteins**
Zinc alpha 2-glycoprotein precursor (ZA2G)	↓	-2.87	(151)	87	28	0008283 : cell proliferation; 0050789 : regulation of biological process; 0007155 : cell adhesion; 0006629 : lipid metabolic process; 0002376 : immune system process; 0050896 : response to stimulus	0008289: lipid binding; 0031406: carboxylic acid binding; 0016787: hydrolase activity; 0005215: transporter activity; 0005515: protein binding	

Table 4.1 contd

Protein	*Up or Down regulation*	*Expression in non-survivors*	*References*	*PubMed References*	*Spot*	*Gene Ontology (GO)*		*Category*
						Process	**Function**	
Lysosomal Acid Phosphatase (PPAL)	↑	2.68	(152)	1021	15	0050896: response to stimulus; 0007033: vacuole organization; 0032501: multicellular organismal process; 0032502: developmental process	0005515: protein binding; 0016787: hydrolase activity	**Phosphatases**
Serine/threonine/tyrosine-interacting-like protein 1 (STYL1)	↓	-2.24	(153)	6	19	0007154: cell communication; 0050789: regulation of biological process; 0006464: protein modification process; 0006793: phosphorus metabolic process	0016787: hydrolase activity; 0005515: protein binding	
Serine/Threonine phosphatase 2-alpha 65K regulatory chain (2AAA)	↓	-2.68	(154)	179	18	0044255: cellular lipid metabolic process; 0007154: cell communication; 0050789: regulation of biological process; 0006260: DNA replication; 0006464: protein modification process; 0006793: phosphorus metabolic process; 0006396: RNA processing; 0006350: transcription; 0065003: macromolecular complex assembly; 0050896: response to stimulus; 0007155: cell adhesion	0005488: binding; 0030234: enzyme regulator activity; 0003823: antigen binding; 0005515: protein binding	
Ras-related protein Rab-37 (RAB37)	↓	-3.82	(155)	8	31	0015031: protein transport; 0007154: cell communication; 0050789: regulation of biological process; 0006810: transport	0000166: nucleotide binding	**G proteins of the Ras family**
Haptoglobin (HPT)	↑	2.91	(156)	7005	8	0050896: response to stimulus; 0065008: regulation of biological quality; 0006508: proteolysis	0003824: catalytic activity; 0005515: protein binding; 0008233: peptidase activity	**Globins**
Tryptophan/serine protease (YHOO4)	↑	3.07	(157)	2	27	0006508: proteolysis	0008233: peptidase activity; 0003824: catalytic activity; 0016787: hydrolase activity	**Proteases**
Brain-enriched guanylate kinase-associated protein (BEGIN)	↓	-5.01	(158)	4	4	-	0005515: protein binding	**Kinases**
Pyruvate kinase (KPYM)	↑	2.06	(159)	2968	29	0005975: carbohydrate metabolic process; 0006066: alcohol metabolic process; 0006091: generation of precursor metabolites and energy; 0043170: macromolecule metabolic process; 0016265: death	0003824: catalytic activity; 0043167: ion binding; 0000166: nucleotide binding; 0001882: nucleoside binding; 0016740: transferase activity; 0005515: protein binding	

Table 4.1 contd

Protein	*Up or Down regulation*	*Expression in non-survivors*	*References*	*PubMed References*	*Spot*	*Gene Ontology (GO)*		*Category*
						Process	**Function**	
Fibrinogen (FIBB)	↓	-2.51	(160)	35467	26	0050817: coagulation; 0050878: regulation of body fluid levels; 0050896: response to stimulus; 0001775: cell activation; 0007154: cell communication; 0050789: regulation of biological process; 0051258: protein polymerization	0005515: protein binding; 0043498: cell surface binding	**Membrane and skeletal proteins**
Fascin (FSCN1)	↑	3.02	(161)	262	20	0006996: organelle organization; 0008283: cell proliferation; 0022607: cellular component assembly	0005515: protein binding	
Mutant desmin (DESM)	↑	2.71	(162)	94	33	-	0005198: structural molecule activity; 0005515: protein binding	
Ankyrin protein 42 (ANR42)	↓	-2.51	(163)	30	23	-	GO:0005515; Protein binding	
Phosphoglucomutase-like protein 5 (PGM5)	↓	-4.87	(164)	21	3	0005975: carbohydrate metabolic process; 0006066: alcohol metabolic process; 0007155: cell adhesion	0043167: ion binding; 0016853: isomerase activity; 0005198: structural molecule activity; 0005515: protein binding	**Metabolic enzymes**
Glyoxalase domain-containing protein 4 (GLOD4)	↓	-3.57	(165)	2	5	-	-	
26S protease regulatory subunit 7 (PRS7)	↑	2.46	(166)	14	13	0044419: interspecies interaction between organisms; 0019538: protein metabolic process; 0043285: biopolymer catabolic process; 0007049: cell cycle; 0065007: biological regulation; 0006508: proteolysis	0000166: nucleotide binding; 0001882: nucleoside binding; 0016787: hydrolase activity; 0005515: protein binding	
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9, mitochondrial (CIA30)	↑	2.76	(167)	197	30	0065003: macromolecular complex assembly; 0006119: oxidative phosphorylation; 0022900: electron transport chain; 0045333: cellular respiration	0005515: protein binding	

Table 4.1 contd.

Protein	*Up or Down regulation*	*Expression in non-survivors*	*References*	*PubMed References*	*Spot*	*Gene Ontology (GO)*		*Category*
						Process	**Function**	
Alpha 1 antitrypsin precursor (A1AT)	↓	-2.97	(168)	10202	7	0050817: coagulation; 0050878: regulation of body fluid levels; 0050896: response to stimulus; 0051704: multi-organism process	0008233: peptidase activity; 0030234: enzyme regulator activity; 0016787: hydrolase activity; 0005515: protein binding	
Nuclear localized factor 1 (NLF1)	↓	-2.09	(169)	2	22	-	-	
Human Serum Albumin (ALBU)	↓	-2.01	(170)	53462	32	0006810: transport; 0007154: cell communication; 0050896: response to stimulus; 0016265: death; 0032502: developmental process; 0050789: regulation of biological process; 0031640: killing of cells of another organism; 0044419: interspecies interaction between organisms; 0065008: regulation of biological quality; 0051640: organelle localization	0003676: nucleic acid binding; 0016209: antioxidant activity; 0008144: drug binding; 0043167: ion binding; 0005496: steroid binding; 0019842: vitamin binding; 0048037: cofactor binding; 0008289: lipid binding; 0031406: carboxylic acid binding; 0005488: binding; 0019825: oxygen binding; 0005515: protein binding	**Others**
Cancer associated gene 1 (CAGE)	↓	-2.45	(171)	4	6	-	-	**Unknown**

The table above shows the functional clustering of the proteins identified from mass spectrometry. The expression of each protein was based on the normalised volume of each of the protein spots. An example reference is shown alongside the amount of literature in PubMed on the protein. The GO slim codes for each of the proteins are provided for Biological Process and Molecular Function. The classification was made simpler according to literature on potential associations with meningitis.

(3) ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The protein Pearson (FASTA) sequences generated from mass spectrometry were inputted into ClustalW (122). ClustalW allows the multiple alignments of protein sequences. The basic information they provide is the identification of conserved sequence regions. This is very useful in designing experiments to test and modify the function of specific proteins, in predicting the function and structure of proteins and in identifying new members of protein families. A phylogram was generated (a branching diagram (tree) that is assumed to be an estimate of a phylogeny). The branch lengths were proportional to the amount of inferred evolutionary change.

4.3 Results

Protein spots which were found to be differentially expressed in normal and clinically diagnosed meningitis CSF were excised and analysed with MALDI TOF and LC-MS/MS.

4.3.1 Common Proteins of Normal and Meningitis CSF

The majority of proteins identified in normal CSF which matched to meningitis CSF (n = 20) included immunoglobulin G (IgG, heavy and light chains, 40%) and serum albumin (20%). Other common proteins found included, ceruloplasmin, transthyretin, α-1-antichymotrypsin, apolipoprotein-a-1, apolipoprotein-a-4 and haptoglobin.

4.3.2 Protein Differences between Non-survivors and Survivors

Protein Identification

34 proteins were identified as showing a minimum 2-fold expression difference in non-survivors compared to survivors. The protein spot differences identified are given in full in Table 4.1 with corresponding expression given in appendix E. Protein differences with the largest fold expression included: Chitotriosidase (CHIT1), Serotransferrin (TRFE),

Brain-enriched guanylate kinase-associated (BEGIN), Complement C3 precursor (CO3), phosphoglucomutase-like protein 5 (PGM5), Cleavage stimulation factor 64 kDa subunit tau variant (CSTFT), Beta-2-glycoprotein 1 precursor (APOH), Solute carrier family 25 (member 16, GDC), Fascin (Singed-like protein, FSCN1), complement C1q tumour necrosis factor-related protein 9 (C1QT9), tryptophan/serine protease (YH004), Rab-37 and glyoxalase domain-containing protein 4 (GLOD4).

Functional Clustering

The proteins were cross-referenced using there UniProtKB/ Swiss-Prot accession numbers. The accession numbers were inputted into the PIR database and searched against the GO database. Most of the proteins identified had multiple processes and functions, which made analysis complex (Figure 4.2 and 4.3). Thus by cross referencing the proteins with known aspects of pneumococcal meningitis, the identified protein spots were functionally clustered into the following groups: Membrane and skeletal proteins (n = 4), transporters (n = 2), glycoproteins (n = 2), G proteins of the Ras family (n = 1), metabolic enzymes (n = 4), cellular defence proteins (n = 3), globins (n = 1), kinases (n = 2), phosphatases (n = 3), chaperone (n = 1), protease (n = 1), translation proteins (n = 6), others (n = 3) and proteins of unknown function (n = 1) (Figure 4.4).

Evolutionary Clustering

To determine if there was any evolutionary relationship between the proteins identified a phylogram tree was constructed for the proteins with ClustalW, as shown in figure 4.5. Here we showed that there were 9 groups of close ancestral links between the protein sequences. These links included; group 1 (GLOD4, GDC, PPAL and ZIK1), group 2 (CHIT1, ALBU, and A1AT), group 3 (FSCN1, HNRPC, NLF1, 2AAA, RN112, RXRG, CO3, PRS7, RAB37 and ZA2G), group 4 (FIBB, TRFE, CIA30 and BEGIN), group 5 (CSTFT, APOH and YH004), group 6

Table 4.1 Functional clustering of identified proteins

Protein	*Up or Down regulation*	*Expression in non-survivors*	*References*	*PubMed References*	*Spot*	*Gene Ontology (GO)*		*Category*
						Process	**Function**	
Complement C3 precursor (CO3)	↓	-4.77	(138)	17787	1	0002376: immune system process; 0050789: regulation of biological process; 0050896: response to stimulus; 0051604: protein maturation; 0016044: membrane organization; 0016192: vesicle-mediated transport; 0007154: cell communication	0005515: protein binding; 0030234: enzyme regulator activity	
Chitotriosidase (CHIT1)	↑	5.14	(139)	228	9	0006032: Chitin catabolic process; 0006955: Immune response; 0009617: Response to bacterium	0008061; Chitin binding; 0004568; Chitinase activity	**Cellular defence**
Complement C1q tumor necrosis factor-related protein 9 (C1QT9)	↑	3.42	(140)	65	24	0008152: metabolic process; 0005975: carbohydrate metabolic process; 0050896: response to stimulus; 0051704: multi-organism process; 0002376: immune system process; 0005976: polysaccharide metabolic process; 0009308: amine metabolic process; 0043285: biopolymer catabolic process	0003824: catalytic activity; 0016787: hydrolase activity; 0001871: pattern binding; 0030246: carbohydrate binding; 0043167: ion binding	
T-complex protein 1 subunit zeta (TCPZ)	↓	-2.75	(141)	135	2	0019538 : protein metabolic process; 0006457 : protein folding	0000166 : nucleotide binding; 0001882 : nucleoside binding; 0005515 : protein binding	**Chaperones**
Serotransferrin (TRFE)	↓	-5.47	(142)	19002	21	0006810 : transport; 0006811 : ion transport; 0065008 : regulation of biological quality	0043167 : ion binding; 0005515 : protein binding	**Transporters**
Solute carrier family 25 (member 16) (GDC)	↑	3.97	(143)	38	34	0006810: transport.	0015300: solute antiporter activity.	

Table 4.1 contd.

Protein	*Up or Down regulation*	*Expression in non-survivors*	*References*	*PubMed References*	*Spot*	*Gene Ontology (GO)*		*Category*
						Process	**Function**	
Retinoic acid receptor RXR-gamma (RXRG)	↓	-2.46	(144)	431	10	0006350: transcription; 0050789: regulation of biological process; 0016070: RNA metabolic process; 0032501: multicellular organismal process; 0032502: developmental process	0005496: steroid binding; 0003676: nucleic acid binding; 0004871: signal transducer activity; 0030528: transcription regulator activity; 0043167: ion binding	**Transcription and Translation**
Cleavage stimulation factor (CSTFT) 64 kDa subunit, tau variant (CSTFT)	↑	3.30	(145)	533	11	0006396: RNA processing	0003676: nucleic acid binding; 0000166: nucleotide binding	
Heterogeneous nuclear ribonucleoproteins C1/C2 (HNRPC)	↑	2.46	(146)	1665	12	0006396: RNA processing	0005515: protein binding; 0003676: nucleic acid binding; 0000166: nucleotide binding	
Zinc finger protein 179 (RN112)	↑	2.02	(147)	23	16	0006457: Protein folding.	0016787: hydrolase activity; 0005515: protein binding; 0000166: nucleotide binding; 0043167: ion binding	
Zinc finger protein 1 (ZIK1)	↓	-2.02	(148)	5	17	0006350: transcription; 0050789: regulation of biological process; 0016070: RNA metabolic process	0003676: nucleic acid binding; 0043167: ion binding	
Eukaryotic translation initiation factor 2, subunit 2 beta (EIF2S2)	↓	-2.18	(149)	1513	25	0006412: translation	0003676: nucleic acid binding	
Beta-2-glycoprotein 1 precursor (APOH)	↑	3.61	(150)	1709	14	0050789: regulation of biological process; 0050817: coagulation; 0050878: regulation of body fluid levels; 0050896: response to stimulus; 0065007: biological regulation; 0016265: death; 0032502: developmental process; 0006928: cell motion; 0040011: locomotion; 0006662: glycerol ether metabolic process; 0044255: cellular lipid metabolic process; 0032501: multicellular organismal process; 0008283: cell proliferation	0001871: pattern binding; 0030246: carbohydrate binding; 0043498: cell surface binding; 0008289: lipid binding; 0030234: enzyme regulator activity; 0005515: protein binding	**Glycoproteins**
Zinc alpha 2-glycoprotein precursor (ZA2G)	↓	-2.87	(151)	87	28	0008283 : cell proliferation; 0050789 : regulation of biological process; 0007155 : cell adhesion; 0006629 : lipid metabolic process; 0002376 : immune system process; 0050896 : response to stimulus	0008289: lipid binding; 0031406: carboxylic acid binding; 0016787: hydrolase activity; 0005215: transporter activity; 0005515: protein binding	

Table 4.1 contd

Protein	*Up or Down regulation*	*Expression in non-survivors*	*References*	*PubMed References*	*Spot*	*Gene Ontology (GO)*		*Category*
						Process	**Function**	
Lysosomal Acid Phosphatase (PPAL)	↑	2.68	(152)	1021	15	0050896: response to stimulus; 0007033: vacuole organization; 0032501: multicellular organismal process; 0032502: developmental process	0005515: protein binding; 0016787: hydrolase activity	**Phosphatases**
Serine/threonine/tyrosine-interacting-like protein 1 (STYL1)	↓	-2.24	(153)	6	19	0007154: cell communication; 0050789: regulation of biological process; 0006464: protein modification process; 0006793: phosphorus metabolic process	0016787: hydrolase activity; 0005515: protein binding	
Serine/Threonine phosphatase 2-alpha 65K regulatory chain (2AAA)	↓	-2.68	(154)	179	18	0044255: cellular lipid metabolic process; 0007154: cell communication; 0050789: regulation of biological process; 0006260: DNA replication; 0006464: protein modification process; 0006793: phosphorus metabolic process; 0006396: RNA processing; 0006350: transcription; 0065003: macromolecular complex assembly; 0050896: response to stimulus; 0007155: cell adhesion	0005488: binding; 0030234: enzyme regulator activity; 0003823: antigen binding; 0005515: protein binding	
Ras-related protein Rab-37 (RAB37)	↓	-3.82	(155)	8	31	0015031: protein transport; 0007154: cell communication; 0050789: regulation of biological process; 0006810: transport	0000166: nucleotide binding	**G proteins of the Ras family**
Haptoglobin (HPT)	↑	2.91	(156)	7005	8	0050896: response to stimulus; 0065008: regulation of biological quality; 0006508: proteolysis	0003824: catalytic activity; 0005515: protein binding; 0008233: peptidase activity	**Globins**
Tryptophan/serine protease (YHOO4)	↑	3.07	(157)	2	27	0006508: proteolysis	0008233: peptidase activity; 0003824: catalytic activity; 0016787: hydrolase activity	**Proteases**
Brain-enriched guanylate kinase-associated protein (BEGIN)	↓	-5.01	(158)	4	4	-	0005515: protein binding	**Kinases**
Pyruvate kinase (KPYM)	↑	2.06	(159)	2968	29	0005975: carbohydrate metabolic process; 0006066: alcohol metabolic process; 0006091: generation of precursor metabolites and energy; 0043170: macromolecule metabolic process; 0016265: death	0003824: catalytic activity; 0043167: ion binding; 0000166: nucleotide binding; 0001882: nucleoside binding; 0016740: transferase activity; 0005515: protein binding	

Table 4.1 contd

Protein	*Up or Down regulation*	*Expression in non-survivors*	*References*	*PubMed References*	*Spot*	*Gene Ontology (GO)*		*Category*
						Process	**Function**	
Fibrinogen (FIBB)	↓	-2.51	(160)	35467	26	0050817: coagulation; 0050878: regulation of body fluid levels; 0050896: response to stimulus; 0001775: cell activation; 0007154: cell communication; 0050789: regulation of biological process; 0051258: protein polymerization	0005515: protein binding; 0043498: cell surface binding	**Membrane and skeletal proteins**
Fascin (FSCN1)	↑	3.02	(161)	262	20	0006996: organelle organization; 0008283: cell proliferation; 0022607: cellular component assembly	0005515: protein binding	
Mutant desmin (DESM)	↑	2.71	(162)	94	33	-	0005198: structural molecule activity; 0005515: protein binding	
Ankyrin protein 42 (ANR42)	↓	-2.51	(163)	30	23	-	GO:0005515; Protein binding	
Phosphoglucomutase-like protein 5 (PGM5)	↓	-4.87	(164)	21	3	0005975: carbohydrate metabolic process; 0006066: alcohol metabolic process; 0007155: cell adhesion	0043167: ion binding; 0016853: isomerase activity; 0005198: structural molecule activity; 0005515: protein binding	**Metabolic enzymes**
Glyoxalase domain-containing protein 4 (GLOD4)	↓	-3.57	(165)	2	5	-	-	
26S protease regulatory subunit 7 (PRS7)	↑	2.46	(166)	14	13	0044419: interspecies interaction between organisms; 0019538: protein metabolic process; 0043285: biopolymer catabolic process; 0007049: cell cycle; 0065007: biological regulation; 0006508: proteolysis	0000166: nucleotide binding; 0001882: nucleoside binding; 0016787: hydrolase activity; 0005515: protein binding	
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9, mitochondrial (CIA30)	↑	2.76	(167)	197	30	0065003: macromolecular complex assembly; 0006119: oxidative phosphorylation; 0022900: electron transport chain; 0045333: cellular respiration	0005515: protein binding	

Table 4.1 contd.

Protein	*Up or Down regulation*	*Expression in non-survivors*	*References*	*PubMed References*	*Spot*	*Gene Ontology (GO)*		*Category*
						Process	**Function**	
Alpha 1 antitrypsin precursor (A1AT)	↓	-2.97	(168)	10202	7	0050817: coagulation; 0050878: regulation of body fluid levels; 0050896: response to stimulus; 0051704: multi-organism process	0008233: peptidase activity; 0030234: enzyme regulator activity; 0016787: hydrolase activity; 0005515: protein binding	
Nuclear localized factor 1 (NLF1)	↓	-2.09	(169)	2	22	-	-	
Human Serum Albumin (ALBU)	↓	-2.01	(170)	53462	32	0006810: transport; 0007154: cell communication; 0050896: response to stimulus; 0016265: death; 0032502: developmental process; 0050789: regulation of biological process; 0031640: killing of cells of another organism; 0044419: interspecies interaction between organisms; 0065008: regulation of biological quality; 0051640: organelle localization	0003676: nucleic acid binding; 0016209: antioxidant activity; 0008144: drug binding; 0043167: ion binding; 0005496: steroid binding; 0019842: vitamin binding; 0048037: cofactor binding; 0008289: lipid binding; 0031406: carboxylic acid binding; 0005488: binding; 0019825: oxygen binding; 0005515: protein binding	**Others**
Cancer associated gene 1 (CAGE)	↓	-2.45	(171)	4	6	-	-	**Unknown**

The table above shows the functional clustering of the proteins identified from mass spectrometry. The expression of each protein was based on the normalised volume of each of the protein spots. An example reference is shown alongside the amount of literature in PubMed on the protein. The GO slim codes for each of the proteins are provided for Biological Process and Molecular Function. The classification was made simpler according to literature on potential associations with meningitis.

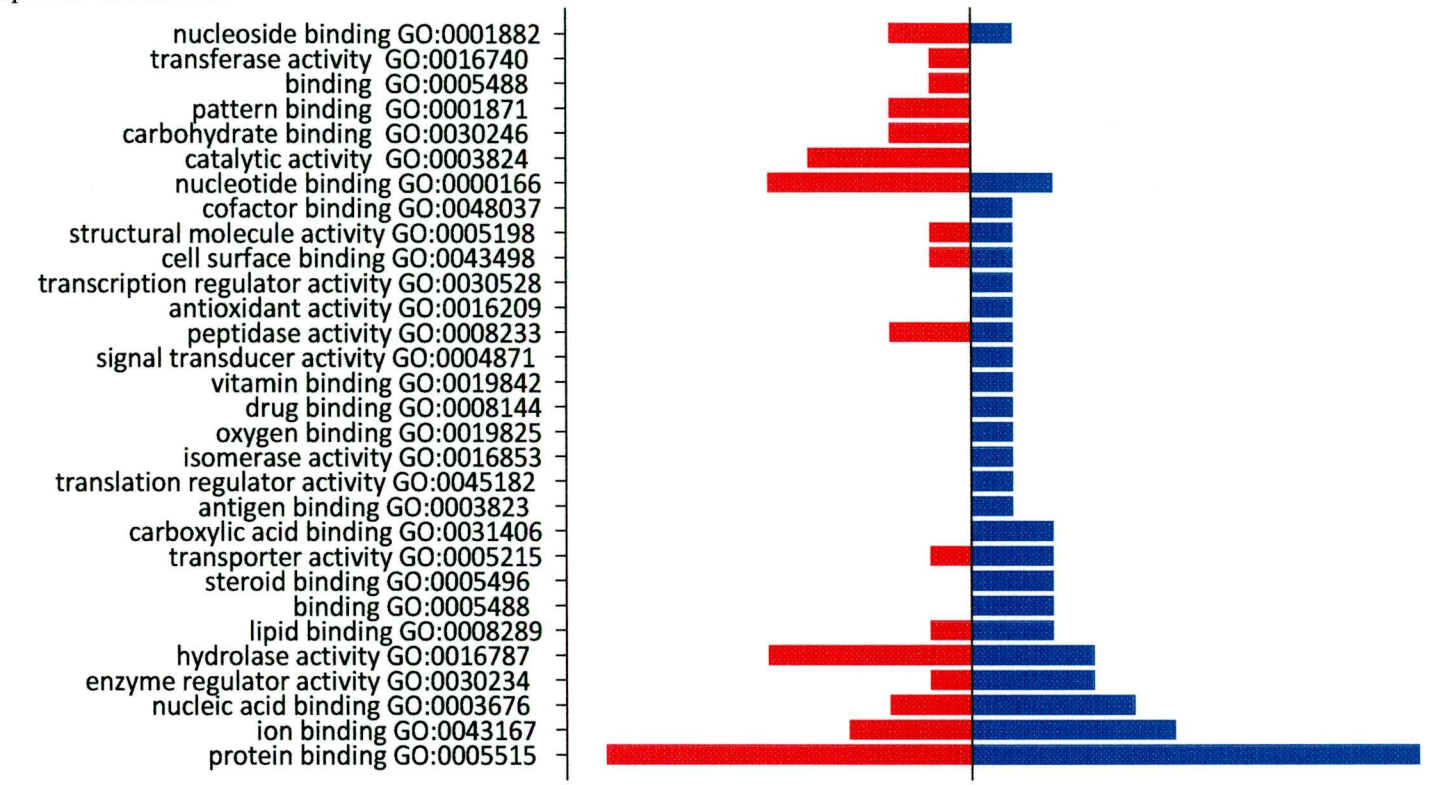

Figure 4.2 GO Molecular functions discovered for all proteins identified. *The GO molecular function codes identified for the proteins are shown on the left panel. The red bands represent upregulated proteins and the blue bands represent downregulated proteins.*

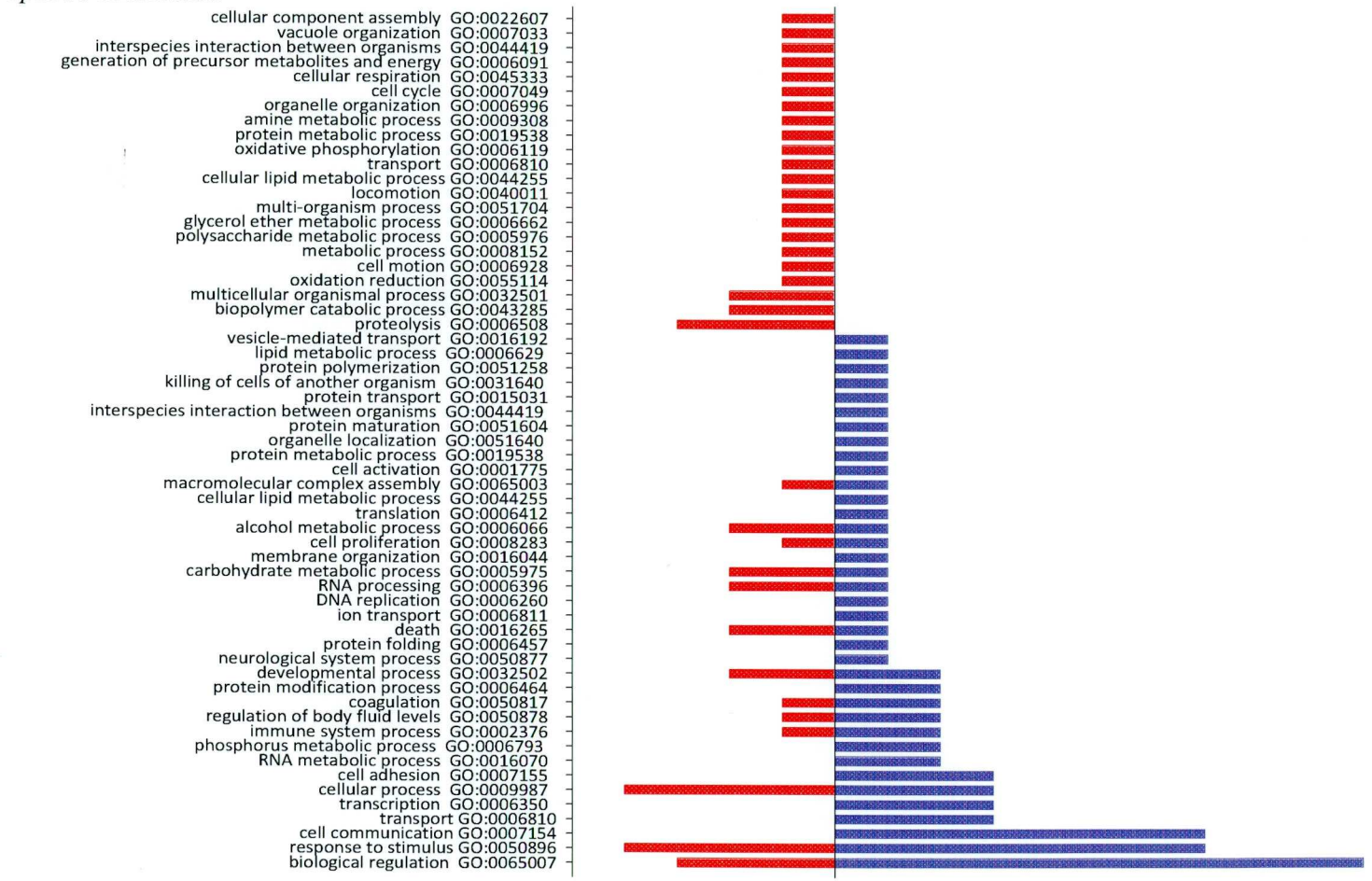

Figure 4.3 GO Biological Processes discovered for all proteins identified. *The GO molecular function codes identified for the proteins are shown on the left panel. The red bands are upregulated proteins and the blue bands are downregulated proteins.*

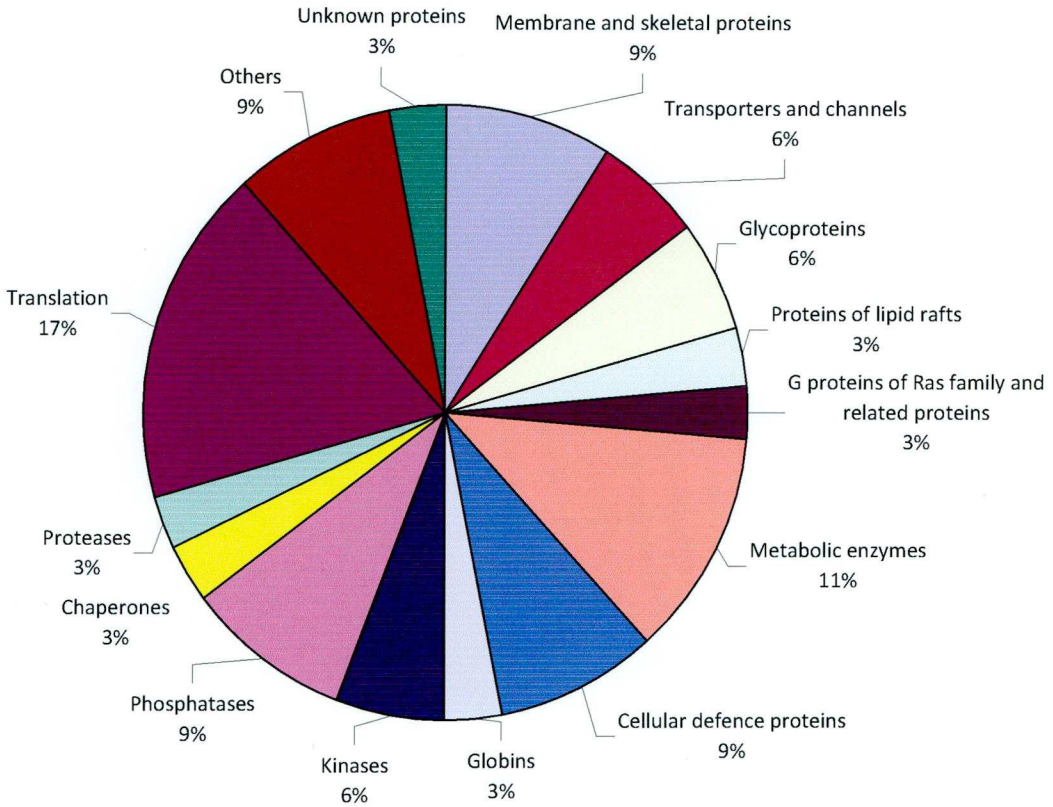

Figure 4.4 Functional Clustering of Proteins Identified by Mass Spectrometry. *Clustering of the proteins identified was made simpler using literature relating possible functions associated with meningitis to the proteins.*

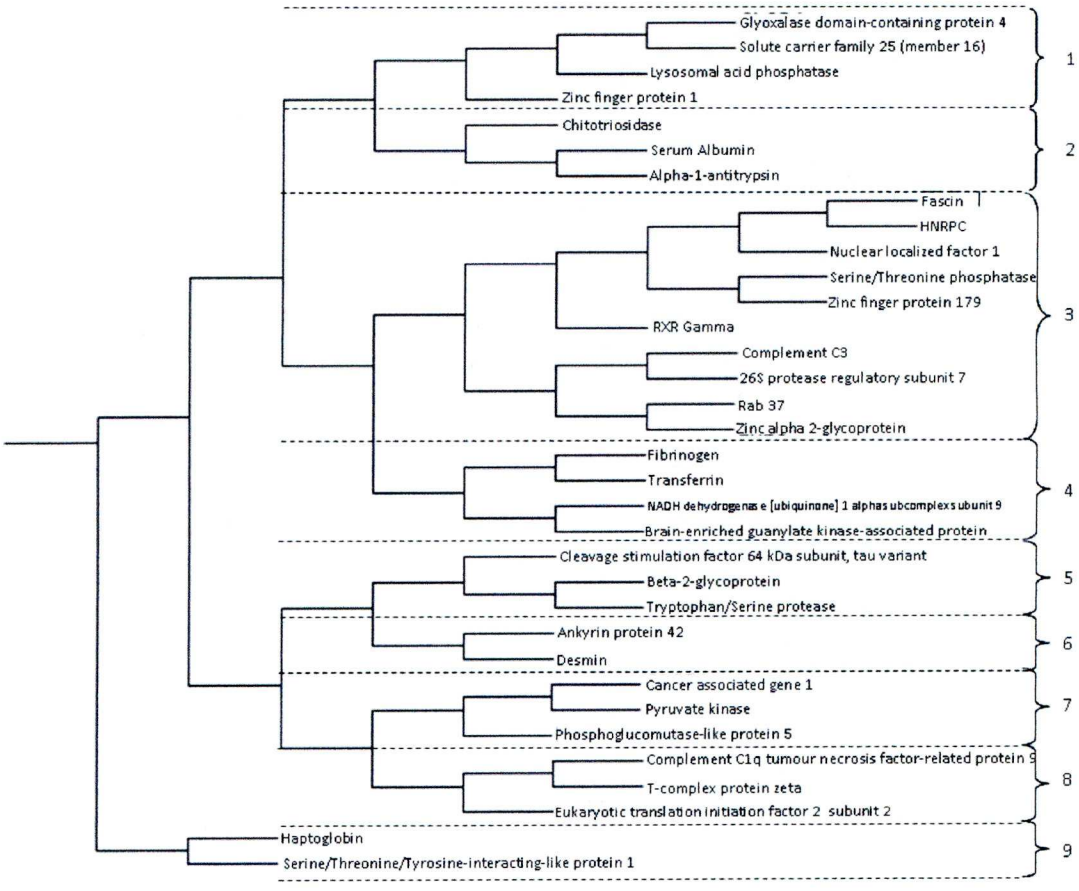

Figure 4.5 Phylogram of Proteins Identified by Mass Spectrometry. *A phylogram is a branching diagram (tree) that is assumed to be an estimate of a phylogeny. The branch lengths are proportional to the amount of inferred evolutionary change. There were 9 groups of proteins with close ancestry. These ancestry links were based on the amino acid sequence homology of the proteins identified.*

(ANR42 and DESM), group 7 (CAGE1, KPYM and PGM5), group 8 (CIQT9, TCPZ and EIF2S2) and group 9 (HPT and STYL1).

4.4 Discussion

In this analysis 34 protein differences were observed between non-survivors and survivors of pneumococcal meningitis. These 34 proteins were subsequently identified with mass spectrometry.

4.4.1 Functional Clustering

Clustering of the proteins revealed that most of the proteins identified were associated with inflammation, metabolism or protein transcription and translation. The GO analysis was able to provide an accumulation of the current information available on the proteins identified. This was useful for identifying a potential association of each protein with meningitis. GO revealed that many of the proteins have multiple functions. Therefore the groupings were reduced using a literature search to focus in on any association of the proteins with pneumococcal infection, meningitis or CSF.

4.4.2 Protein Functions

Most of the proteins identified were likely to be a result of the breakdown of the BBB and may be present in blood plasma. No clear abnormal pathway looked to be altered based on clusters of proteins from a common pathway going up or down. There were no pneumococcal proteins identified in the list of differences between non-survivors and survivors. Surprisingly there were no cytokines also found amongst the protein differences. This may have been due to the high abundance of large host endogenous proteins in the CSF. A brief discussion of the proteins identified is given below (fold difference values were for non-survivors):

Cellular Defence

Chitotriosidase (CHIT1, upregulated 5-fold in non-survivors) degrades chitin and chitotriose, and may participate in the defence against nematodes and other pathogens (172). CHIT1 can be considered an inflammatory protein since it is solely secreted by activated macrophages and may be used as an important predictor of neuronal disease severity (139).

Complement C3 (C3, downregulated 4-fold in non-survivors) is essential for the opsonisation of *S. pneumoniae* (138). C3 plays a central role in the activation of the complement system. Processing by C3 convertase is the central reaction in both classical and alternative complement pathways. C3, aids innate immunity by three main mechanisms: the coating of pathogens with C3b and iC3b, which stimulate phagocytosis and the release of proinflammatory mediators C3a and C5a.

C1q tumour necrosis factor related protein 9 (C1QT9), was identified by Clark et al. in 2008 (154). This protein consists of a C1q domain and also a collagen-like domain. The exact functions are not known, but it may have similar properties to complement and may have inflammatory properties (140).

Membrane and Skeletal Proteins

Several cytoskeletal associated proteins were also found amongst the spot differences. These proteins were most likely linked with the breakdown of the blood-brain barrier or as a result of neuronal cell death; Fascin (FSCN1, 3-fold upregulation in non-survivors) is involved in the assembly of actin filament bundles present in microspikes, membrane ruffles, and stress fibres. In the brain, FSCN1 expression has been localised to neurons, glial cells, and endothelial cells. It has been found to

be associated with filamentous actin condensation during invasion of the CNS by *E. coli* (161). Therefore it was possible that a similar mechanism occurs during pneumococcal invasion.

Desmin (DESM, 2-fold upregulation in non-survivors) is most commonly found in muscle cells and is a member of the cytoskeleton of muscle cells. It has been detected in CSF before as it is found in the endothelial cells of the wall of the meninges (162).

Fibrinogen (FIBB, 2-fold downregulated in non-survivors) has a double function; yielding monomers that polymerise into fibrin and acting as a cofactor in platelet aggregation. Bacteria such as *S. agalactiae* have been shown to adhere to or invade tissues by utilizing fibrinogen (160). The downregulation of this protein in non-survivors was most likely a result of severe depletion of fibrinogen due to the blood clotting cascade (173).

Ankyrin repeat domain-containing protein 42 (ANR42, 2-fold down regulation in non-survivors) is a member of a group of proteins which contain an ankyrin repeat. It was first discovered as a repeated sequence in yeast cell-cycle regulation proteins. Ankyrin repeats are common in signalling proteins, and appear to be general protein–protein interaction motifs (163). The exact functions are not known but may have an association with protein binding during the cell cycle.

Metabolic Enzymes

Phosphoglucomutase like protein 5 (PGM5 or Aciculin, downregulated 4-fold in non-survivors) is a component of adherens-type cell-cell and cell-matrix junctions (164). It has not been previously identified in the brain, however, PGM5 is part of this dystrophin–utrophin complex (174). In the brain, utrophin is present in the choroid plexus

epithelium and vascular endothelial cells. The short C-terminal isoform of dystrophin (Dp71) is localized in the glial end-feet surrounding blood vessels (175). Both proteins have been localized in specific types of neurons in the brain and were most likely to be the source of PGM5 in CSF either as a result of breakdown of the BBB or direct neuronal damage. Enzymatic activity associated with PGM5 has not been established therefore the reason for the downregulation of this protein was not known.

Glyoxalase domain-containing protein 4 (GLOD4, downregulated 3-fold in non-survivors) was found during the cloning and characterisation of a novel gene (C17orf25) from the deletion region on chromosome 17p13.3 in hepatocellular carcinoma (176). It is a member of the glyoxalase system, a set of enzymes that play a role in detoxification of methylglyoxal and the other reactive aldehydes produced during metabolism (177). The protein methyl glyoxal has been shown to induce neurotoxicity through the impairment of detoxification pathways and depletion of reduced glutathione (165).

The 26S protease regulatory subunit 7 (PRS7, upregulated 2 fold in non-survivors) is involved in the ATP-dependent degradation of ubiquitinated proteins. The regulatory (or ATPase) complex confers ATP dependency and substrate specificity to the 26S complex. It is involved in many important biological processes such as cell cycle progression, apoptosis, and DNA repair (166).

NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9, mitochondrial (CIA30, upregulated 2-fold in non-survivors) is an accessory subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I), that is believed to be not involved in catalysis. Complex I functions in the transfer of electrons from NADH to

the respiratory chain. The immediate electron acceptor for the enzyme is believed to be ubiquinone. This molecule has been shown to be expressed in mice upon infection by *S. pneumoniae* (178).

Transcription and Translation Proteins

Retinoic acid X receptor gamma (RXRG, 2-fold downregulation in non-survivors) is a member of the retinoid X receptor (RXR) family. These receptors are involved in the expression of anti-inflammatory activity by immune cells and mediate the cellular effects of retinoid compounds (144). In mice, vitamin A deficiency is associated with increased T-cell production of IFN-γ and decreased production of IL-4 and IL-10. This could potentially suggest an association between vitamin A deficiency and survival of meningitis.

Cleavage stimulation factor 64 kDa subunit, tau variant (CSTFT upregulated 3-fold in non-survivors) plays a significant role in mRNA polyadenylation and is directly involved in binding to pre-mRNAs. Upregulation in non-survivors was most likely due to a rapid increase in the production of protein possibly linked to the severity of the disease (145).

Heterogeneous nuclear ribonucleoproteins C1/C2 (HNRPC, upregulated 2-fold in non-survivors) binds pre-mRNA and nucleates the assembly of 40S hnRNP particles. It plays a role in the early steps of spliceosome assembly, pre-mRNA splicing and interacts with poly-U tracts in the 3'-UTR or 5'-UTR of mRNA and modulates the stability and the level of translation of bound mRNA molecules (146).

Zinc finger protein 179 (RN112, upregulated 2-fold in non-survivors) is predominantly expressed in the brain. This protein encodes a member of the RING finger protein family of transcription factors and is

associated with neuronal differentiation. It has been implicated in a variety of functions such as transcriptional regulation, DNA repair, site-specific recombination and signal transduction (147). The upregulation of this protein in non-survivors was most likely a result of neurogenesis as result of neuronal cell death (179).

Zinc finger protein 1 (ZIK1, upregulated 2-fold in non-survivors) has been found to be associated with transcriptional repression (148). It interacts with heterogeneous ribonucleoprotein K (HNRPK), one of the major pre-mRNA-binding proteins.

Eukaryotic translation initiation factor 2 (EIF2S2, 2-fold downregulation in non-survivors) is responsible for one of the earliest steps in the initiation of protein synthesis. The upregulation in survivors may be due to an association with an increase in protein production during the disease (149).

Phosphatases

Lysosomal Acid Phosphatase (PPAL, upregulated 2-fold in non-survivors) is a member of a family of distinct isoenzymes which hydrolyse orthophosphoric monoesters to alcohol and phosphate. Lysosomal enzymes are released when lysosomes are exposed to free radical lipid peroxidation (152).

Serine/Threonine phosphatase (2AAA, upregulated 2-fold in non-survivors) was genetically identified by Walter et al. in 1989 (180). 2AAA has been shown to be associated with neuroprotection (181).

Serine/threonine/tyrosine-interacting-like protein 1 (STYL1, downregulated 2-fold in non-survivors) also known as Map kinase phosphatase-like protein (153) has been found to associate with the innate immune responses. MAP kinase cascades are crucial in the biosynthesis

of proinflammatory cytokines (157). MAP kinase phosphatase has been found to regulate MAP kinase in a number of cell signalling pathways, thus it may be providing a neuroprotective effect against MAP kinase (182).

Transport Proteins

Solute carrier protein 25 member 16 (upregulated 3-fold in non-survivors) is a mitochondrial carrier protein. Mitochondrial carrier proteins, which are localized in the inner membrane, facilitate the rapid transport and exchange of molecules between the cytosol and the mitochondrial matrix space. Members of the solute carrier family play an important role in the efflux transport of the blood brain barrier, especially for organic anions and xenobiotic compounds, effectively functioning as a detoxification system in the brain (143). Its upregulation may be associated with the release of the pore forming toxin pneumolysin from pneumococci which is known to degrade the mitochondrial membrane as well as the cell membrane leading to neuronal death.

Transferrin (TRFE, downregulated 5-fold in non-survivors) is an iron binding transport protein with antibacterial properties. It is responsible for the transport of iron from sites of absorption and haem degradation to those of storage and utilization. TRFE may also have a further role in stimulating cell proliferation (142). The breakdown of the BBB was the most likely source. However, the downregulation of this protein suggest an association with iron which is essential for the nutrient growth of organisms. The ability to acquire iron under low-iron conditions is related to the virulence of a variety of bacterial pathogens. The CSF is a low region of iron therefore *Streptococcus pneumoniae* have tended to acquire iron through pneumococcal uptake proteins such PiA and PiU. Therefore it was possible that pneumococci were utilizing

transferrin as a source of iron in CSF leading to its reduction in non-survivors (183).

Glycoproteins

Two glycoproteins were discovered; Beta-2-glycoprotein I (APOH, implicated in processes such as coagulation and atherosclerosis) was most likely upregulated (3-fold) in non-survivors as a direct result of blood clotting during the breakdown of the blood-brain barrier (150).

Zinc α–2-glycoprotein (2-fold downregulation in non-survivors) is secreted in various body fluids where it is involved preferentially in depletion of fatty acids from adipose tissues, subsequently named as lipid-mobilising factor (151).

Kinases

Two kinases were found; Pyruvate kinase (KPYM 5-fold downregulation in non-survivors) which is an enzyme involved in glycolysis. Pyruvate kinase has previously been associated with changes during neurological disease (159).

Brain-enriched guanylate kinase-associated protein (BEGIN, upregulated 2-fold in non-survivors) was first identified by Deguchi et al. in 1998 (158) as a novel post synaptic density component associated with the PSD-95/synapse associated protein 90 (184). It is found in many neurons and in particular those of the hippocampus. Its presence in CSF was most likely a result of cellular damage. However the observation of reduced expression in non-survivors suggests it may have an alternate unknown role.

Other Proteins

T-complex protein 1 subunit zeta (TCPZ, 2-fold downregulation in non-survivors) is a chaperone protein discovered in humans by Lewis et

al. in 1992 and identified as the human equivalent of the bacterial groEL protein (141). TCPZ is a cytosolic protein associated with the Chaperonins and the heat shock protein (185) responsible for tissue and protein repair. Thus down regulation of this protein may have had a detrimental effect on survival.

Alpha 1 antitrypsin precursor (A1AT, downregulated 2-fold in non-survivors) is an inhibitor of serine proteases. Its primary target is elastase, but it also has a moderate affinity for plasmin and thrombin (186). It Inhibits trypsin, chymotrypsin and plasminogen activator, and the aberrant form inhibits insulin-induced Nitric Oxide synthesis in platelets, decreases coagulation time and has proteolytic activity against insulin and plasmin. It is associated with inflammation, and this particular protein has been found to be upregulated in the lungs of humans and mice with severe pneumonia (168;186).

Nuclear localized factor 1 (NLF1, downregulated 2-fold in non-survivors), identified by Warton et al 2004, may play a role in endothelial cell inflammation, potentially by changing the architecture of endothelial cells with effects on vascular permeability (169). It is specifically expressed in endothelial cells and is up-regulated by pro-inflammatory cytokines.

Human Serum Albumin (ALBU, downregulated 2-fold in non-survivors) maintains oncotic pressure, and is used for transportation e.g. thyroid hormones, fat soluble hormones, fatty acids to the liver, unconjugated bilirubin. It also competitively binds calcium ions (Ca^{2+}) and buffers pH (170)

Haptoglobin (HPT, upregulated 2-fold in non-survivors) combines with free plasma haemoglobin, preventing loss of iron through the

kidneys and protecting the kidneys from damage by haemoglobin, while making the haemoglobin accessible to degradation enzymes. It may have a role in the inflammatory response (156).

Tryptophan/serine protease (YHOO4, upregulated 3-fold in non-survivors) is an enzyme that cleaves lys-arg and arg-ser bonds. It activates, in a reciprocal reaction, factor xii after its binding to a negatively charged surface. It has functions which are very similar to trypsin and chymotrypsin (154).

Cancer associated gene 1 (CAGE1, downregulated 2-fold in non-survivors) was discovered by Sacyoung Park et al in 2002 as part of a serological analysis of cDNA expression library (SEREX) to identify cancer-associated genes from sera of patients with lung cancer The exact functions of CAGE1 are not known (171).

The only G protein of the Ras family identified was the Ras-related protein Rab-37 (RAB37, downregulated 3-fold in non-survivors). RAB37 is a protein which belongs to the small GTPase superfamily which regulates cellular functions including signal transduction, cytoskeletal organization and membrane trafficking. These proteins are commonly found in endothelial cells which compose the BBB (155). Therefore this protein most likely leaked into the CSF after BBB breakdown (187).

4.4.3 Application of Mass Spectrometry

In this analysis both MALDI-TOF and LC-MS/MS were efficient techniques in generating data for analysis. PMF was a fast and easy way to identify a protein, with a success rate of about 70-80% using samples of human origin. ESI was more time consuming than PMF but it provided a better analysis for protein mixtures (188). LC-MS/MS can provide a tandem MS/MS protein profile in 1 hr and MALDI-TOF can provide a

PMF instantaneously (188). The limiting factor in the analysis was the digestion efficiency. Once this was optimised the data was generated quickly. These instruments have high sensitivity e.g. MALDI can detect pg quantities of proteins with Mw up to 300 kDa (112). ESI allows analysis of proteins above 130 kDa with a detection limit of 1 pmol. Detection limits in the range of attomoles have also been demonstrated (114). However one of the major issues occurred when the protein spots consisted of more than one protein as it was difficult to identify the smaller proteins through peptide fragments. In the case of MALDI-TOF, it was necessary to use peak deduction to determine the identity of proteins which could have affected the outcome of the search. This was not the case in LC-MS/MS, but due to the high sensitivity of this technique, proteins in the CSF such as immunoglobulin and albumin tended to confound spectra.

In this analysis the spectral data was searched using Mascot as it provided a clear and objective system for protein identification through the Mowse score. One of the major issues with the results was the low scores which were obtained in the analysis in MALDI-TOF. It was difficult to get a score with MALDI-TOF directly. As a result, it was necessary for the use of blanks to remove contamination peptide peaks to create the final peak list for searching. This may have lead to unnecessary modifications in the identification.

The identified peptides are compiled into a protein 'hit list', which is the output of a typical proteomic experiment. Because protein identifications rely on matches with sequence databases, high-throughput proteomics is currently restricted largely to those species for which comprehensive sequence databases are available. Therefore it is possible that pneumococcal proteins were present in the analysis but due to the inferior amount of protein data compared to the human database, many of

the pneumococcal proteins will have been confounded by host protein. There is also a potential for false positive recognition rates. This remains an open problem as how to validate database search results (119). A further confirmation through Western blotting may be required to validate the proteins identified as well as confirm any clinical significance to the proteins identified.

4.5 Conclusions

In this chapter it was found that the use of mass spectrometry was able to discriminate proteins identified in non-survivor and survivor cohorts. These consisted mostly of cellular defence proteins, protein transcription and translation molecules and metabolic proteins. There are a number of proteins which require further analysis to validate the analysis by mass spectrometry and to determine any clinical significance. The next step in this analysis will be to confirm the fold difference in protein levels found by testing a new set of CSF samples by the alternative protein analysis method of Western blot.

CHAPTER 5

IMMUNODETECTION OF HOST PROTEINS IN CSF OF PATIENTS WITH PNEUMOCOCCAL MENINGITIS

5.1 Introduction

The aim of this chapter was to validate the presence of 9 proteins as potential biomarkers associated with survival from pneumococcal meningitis using Western blotting. The proteins selected for analysis were based on literature regarding cell death associated with pneumococcal disease and the protein identification results from chapters 3 and 4.

Three apoptosis markers were selected for analysis. The apoptosis markers analysed included caspase 3, apoptosis inducing factor (AIF) and the marker of necrosis, creatine kinase BB (CKBB).

Six proteins from the list of proteins identified in chapter 4 were selected for Western blot validation. These six proteins consisted of three proteins which were potentially of CSF origin. These three proteins included; retinoic acid X receptor gamma, zinc finger protein 179 and chitotriosidase. The three proteins which were most likely proteins originating from serum included; β-2-glycoprotein, complement C3 (C3) and transferrin.

SDS PAGE and Western blotting are two important and well-known methods for protein characterisation. Although classical Western blotting is typically used for qualitative purposes, it also can be used for quantitative analysis of proteins.

This chapter will aim to test three hypotheses; (1) Classical cell death protein markers can be detected in CSF. (2) The proteins discovered in chapter 3 and 4, and their expression profiles can be validated with Western blotting. (3) These proteins have a predictive value in explaining the outcome of pneumococcal meningitis.

5.2 Materials and Methods

All chemicals were purchased from Sigma Aldrich (Poole, UK), VWR (Lutterworth, UK) or Fisher (Loughborough, UK) unless otherwise stated. General solutions used for the chapter were made using HPLC grade water. The pH of all solutions used in this chapter was adjusted by the addition of 1 M HCl or NaOH.

5.2.1 Patient information

CSF for this Western blot analysis included samples from the rapid death group (n = 40) and the "well" group (n = 40). Control 'normal' CSF was obtained from patients who had been clinically diagnosed as not having meningitis (n = 10). Details on patient data are provided in table 5.1.

5.2.2 Antibodies and Control Markers

Antibodies

Primary antibodies used in this analysis were directed against caspase 3 (9665 and sc-22140), apoptosis inducing factor (AIF, 4642 and sc-9416) from Cell Signaling (Danvers, MA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA). Creatine kinase BB (CKBB, ab38212), zinc finger protein 179 (ab42499), retinoic acid X receptor gamma (ab15518), chitotriosidase (ab72574) and transferrin (ab1223) from Abcam (Cambridge, UK). Beta-2-glycoprotein (MCA2114, Abd Serotec, Oxford, UK) and complement C3 (C7761-1VL, Sigma Aldrich). Secondary antibodies were purchased from Dako (Cambridge, UK), and Nordic Immunology (Tilburg, Netherlands). A summary of the antibodies used in this chapter are listed in Table 5.2.

Table 5.1. Clinical Details of Patients Used in the Host Western Blot Analysis

	Cerebrospinal fluid sample		
	Normal	***Non-survivors***	***Survivors***
	N = 10	N = 40	N = 40
Age - yrs mean (+/- SD)	27.8 (9.5)	30.1 (8.4)	33.3 (11.2)
Male sex - number	3	23	16
GCS - mean (+/- SD)	9.4 (4.7)	10.9 (3.2)	8.4 (3.3)
Mean time to presentation (IQR, hours)	55.2 (12 - 96)	60.2 (15 - 336)	78.6 (10 - 192)
Previous antimicrobials - number	2	7	6
Steroid Treatment – number (placebo)	-	17 (23)	21 (19)
HIV Positive – number (% of those tested)	-	35 (100)	37 (100)
HIV Not known	10	5	3
Survival at day 10 (%)	100	95	98

The table above shows the clinical data collected from patients providing CSF samples that were used in this analysis. All meningitis subjects were diagnosed as having pneumococcal meningitis caused by Streptococcus pneumoniae, all subjects were HIV positive and all samples were collected before any treatment commenced. Normal CSF was obtained from patients who tested negative for meningitis or any other pathogen. The HIV status of the normal patients was not known.

Table 5.2 Antibodies Used in Host Western Blot Analysis

Antibody phase	*Antibody*	*Antibody type*	*Specificity*	*Host*	*Detection*	*Supplier*
Primary	Zinc finger protein 179	Polyclonal	Human	Rabbit	ECL	Abcam
	RXR Gamma	Polyclonal	Human, mouse, hamster	Rabbit	ECL	Abcam
	Chitotriosidase	Polyclonal	Human	Mouse	ECL	Abcam
	β-2-glycoprotein	Monoclonal	Human	Mouse	BCIP/NBT	AbD Serotec
	Transferrin	Polyclonal	Human	Rabbit	DAB	Abcam
	Complement C3	Polyclonal	Human	Goat	DAB	Sigma Aldrich
	AIF	Polyclonal	Human, mouse	Goat	ECL	Santa Cruz Biotechnology
	AIF	Polyclonal	Human	Rabbit	ECL	Cell Signaling
	Caspase 3	Polyclonal	Human	Rabbit	ECL	Santa Cruz Biotechnology
	Caspase 3	Polyclonal	Human	Rabbit	ECL	Cell Signaling
	Creatine kinase BB	Polyclonal	Human	Rabbit	ECL	Abcam
Secondary	HRP conjugate secondary	IgG	Anti goat	Donkey	ECL/DAB	Dako
	HRP conjugate secondary	IgG	Anti rabbit	Goat	ECL/DAB	Nordic immunology
	HRP conjugate secondary	IgG	Anti mouse	Goat	ECL/DAB	Nordic immunology
	AP conjugate secondary	IgG	Anti mouse	Goat	BCIP/NBT	Sigma Aldrich

The table above shows the antibodies used in this analysis along with the corresponding conjugate used in the analysis i.e. either Horseradish peroxidise (HRP) or alkaline phosphatase (AP).

Control markers

The positive control lysate used for caspase 3 was Jurkat apoptosis cells treated with etoposide (9663, Cell Signaling). For AIF, CKBB and RXR gamma the positive control cell lysate was HeLa whole cell lysate (sc-2200, Santa Cruz Biotechnology). For zinc finger protein 179 the positive control lysate used was HepG2 cell lysate (sc-2227, Santa Cruz Biotechnology). For CHIT1 a recombinant CHIT1 protein was used (3559-GH, R and D systems, Minneapolis, MN, USA).

Blood plasma (obtained from a healthy volunteer) was used as a control for complement C3, transferrin and beta-2-glycoprotein. In addition for transferrin a recombinant transferrin protein was also used as a positive control (Sigma Aldrich, 90190).

In addition to using the normal CSF as a negative control the other negative control used was a normal Western blot of the meningitis CSF samples with the secondary antibody only i.e. without any primary antibody under the same conditions as used in the normal Western blot experiments.

5.2.3 CSF Sample Preparation

Preparation of CSF samples is described in detail in chapter 2. Briefly CSF samples were stored at -20°C within an hour of sampling and at -80°C from 24 hrs until analysis.

5.2.4 Western Blot of CSF

The concentration of protein was determined using the Bradford assay as described in chapter 2. Samples were measured in triplicate. CSF was separated and analysed using SDS PAGE followed by Western blot. This method is described in detail in chapter 2. The concentration of protein calculated in this chapter was based off the total protein content

and therefore a more accurate measure would involve the use of purified recombinant proteins for each of the proteins under analysis. For the comparative analysis this had no effect on the final comparison between normal, non-survivors and survivors.

5.2.5 CKBB Confirmation

Following the selection of protein bands of interest, these bands were manually excised from the stained gels and cut into $1mm^3$ slices. The sections were then subjected to in-gel tryptic digestion and analysed with MALDI-TOF and LC-MS/MS as described in chapter 2.

5.2.6 Statistics

Pairwise comparisons were performed using an unpaired t-test with Welch's correction. This was calculated using Prism software (v. 5.0, GraphPad, La Jolla, CA, USA) and R software (R project for statistical computing, free download from http://www.r-project.org/). Charts were plotted using SigmaPlot (v.10, Systat Software Inc, Chicago, IL, USA) and Prism Software.

5.3 Results

5.3.1 Sample Information

80 subjects were selected for analysis. 40 of these CSF samples were from patients who had an excellent recovery and a short clinical course. These subjects all survived with no neurological impairment (median duration of symptoms etc). The remaining 40 subjects had very poor outcome and all died (median duration of admission = 30 days).

5.3.2 CSF Protein Concentration

The concentration of protein in each sample was measured using the Bradford assay. CSF from patients clinically diagnosed with meningitis had a mean protein concentration of 5.92 $mgml^{-1}$ compared to normal

CSF (mean 0.23 $mgml^{-1}$, $p < 0.0001$). The CSF from non-survivors had a protein concentration of 2.20 – 11.30 $mgml^{-1}$ (mean 6.38 $mgml^{-1}$). The CSF from survivors had a protein concentration of 1.12 – 10.89 $mgml^{-1}$ (mean 5.46 $mgml^{-1}$). The mean protein concentration was higher in CSF from non-survivors compared to CSF from survivors but was not significantly different ($p = 0.09$). This is shown in figure 5.1.

5.3.3 Expression of Protein Markers Associated with Apoptosis

CSF Western Blot to Detect Caspase 3

In the CSF Western blot for caspase 3 none of the 90 CSF samples expressed a protein band which correlated with caspase 3 as shown in Figure 5.2A. Caspase 3 was detectable in the positive control at levels of protein as low as 100 $ng\mu l^{-1}$. This positive control band appeared at approximately 35 kDa which corresponds to the Caspase 3 protein. The 17 kDa cleavage product of caspase 3 was also visible in the positive control but was also not detected in the analysis of the 90 CSF samples. This Western blot was repeated with two primary antibodies directed against different regions of the caspase 3 protein structures with the same outcome. Specificity of the antibodies was confirmed using an antigenic blocking peptide. The blocking peptide binds to the active site of the antibody which inhibits the antibody activity leading to quenching of protein expression.

CSF Western Blot to Detect AIF

In the CSF Western blot to detect AIF none of the 90 CSF samples had a band which correlated with AIF as shown in Figure 5.2B. AIF was detectable at levels of protein as low as 100 $ng\mu l^{-1}$. This positive control band appeared at a level consistent with the 77 kDa AIF protein as shown in figure 5.2B.

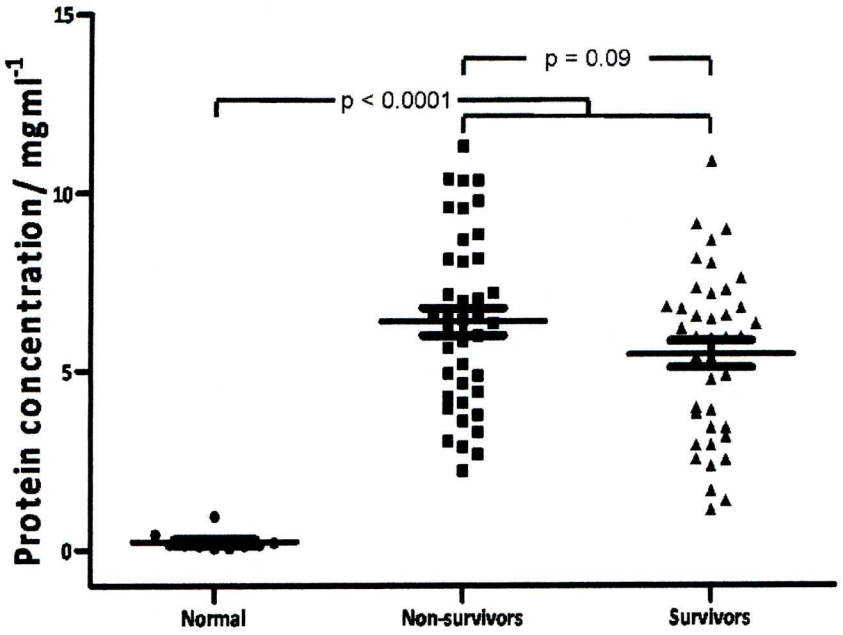

Figure 5.1 Protein concentrations in larger CSF sample size groups. *'Normal' CSF samples had a protein concentration ranging from 0.05 – 0.94 mgml^{-1} (average 0.23 mgml^{-1}). The non-survivors had a protein concentration of 2.20 – 11.30 mgml^{-1} (average 6.38 mgml^{-1}). The survivors had a range of protein concentration of 1.12 – 10.89 mgml^{-1} (average 5.46 mgml^{-1}). The protein concentration in control CSF was significantly less than meningitis affected CSF ($p < 0.0001$). The average protein concentration was higher in non-survivors compared to survivors but was not statistically different ($p = 0.09$).*

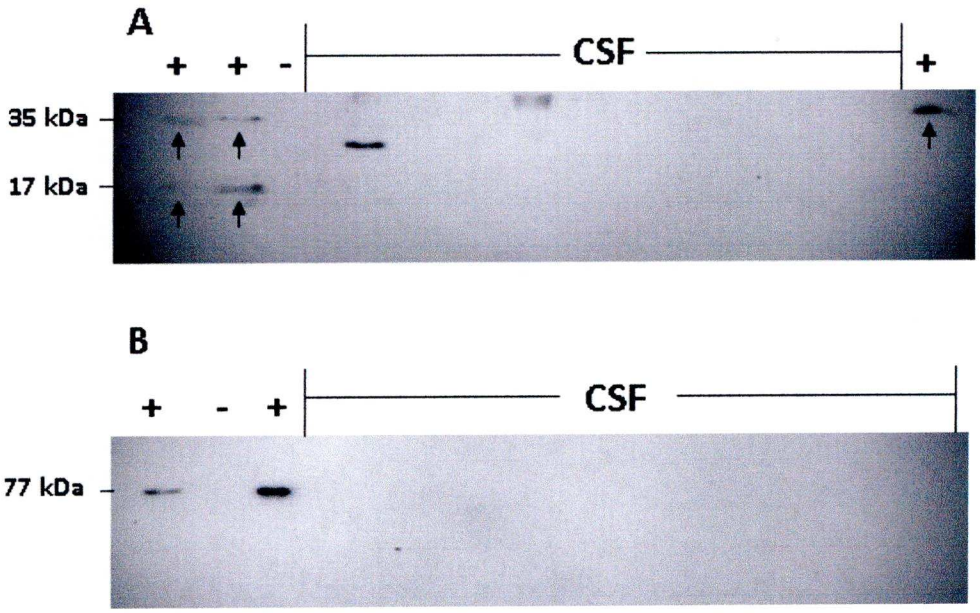

Figure 5.2 Western blot analyses of apoptosis associated proteins caspase 3 and AIF. ***A*** *Western blot of Caspase 3 resulted in negative detection in CSF. Both the 35kDa caspase 3 and cleaved subunits were visible in the positive control.* ***B*** *Western blot of AIF resulted in negative detection of the protein in CSF. The 77kDa protein was visible in the lane containing positive control.*

This Western blot was repeated with two primary antibodies directed against different regions of the AIF protein structures with the same outcome. Specificity of the antibodies was confirmed using an antigenic blocking peptide. The blocking peptide binds to the active site of the antibody which inhibits the antibody activity leading to quenching of protein expression.

CSF Filtration to Expose AIF

When CSF was analysed with SDS PAGE, a significant quantity of protein was also present at the same molecular weight region as AIF. To determine if AIF expression was being inhibited by another protein, a filtration experiment was carried out. Here CSF was spiked with different concentrations of the AIF positive control (5 µl, 2.5 µl and 0 µl of HeLa cell lysate). Half of the samples were filtered using a spin column for proteins above 60 kDa. These samples were then Western blot as described earlier. There was no effect on the outcome of the analysis which indicated that AIF was not being confounded by another protein as demonstrated in figure 5.3.

5.3.4 Expression of Markers Associated with Necrosis

CSF Western Blot to Detect Creatine Kinase BB

Creatine kinase BB (CKBB) was not detected in normal CSF samples. However, CKBB was discovered in over 40% of meningitis CSF samples at varying concentrations as shown in figure 5.4. The mean concentration of CKBB in non-survivors (mean 0.89 $mgml^{-1}$) was higher than the mean concentration of CKBB in survivors (mean 0.19 $mgml^{-1}$). However there was no statistically significant difference ($p = 0.16$) as shown in figure 5.5.

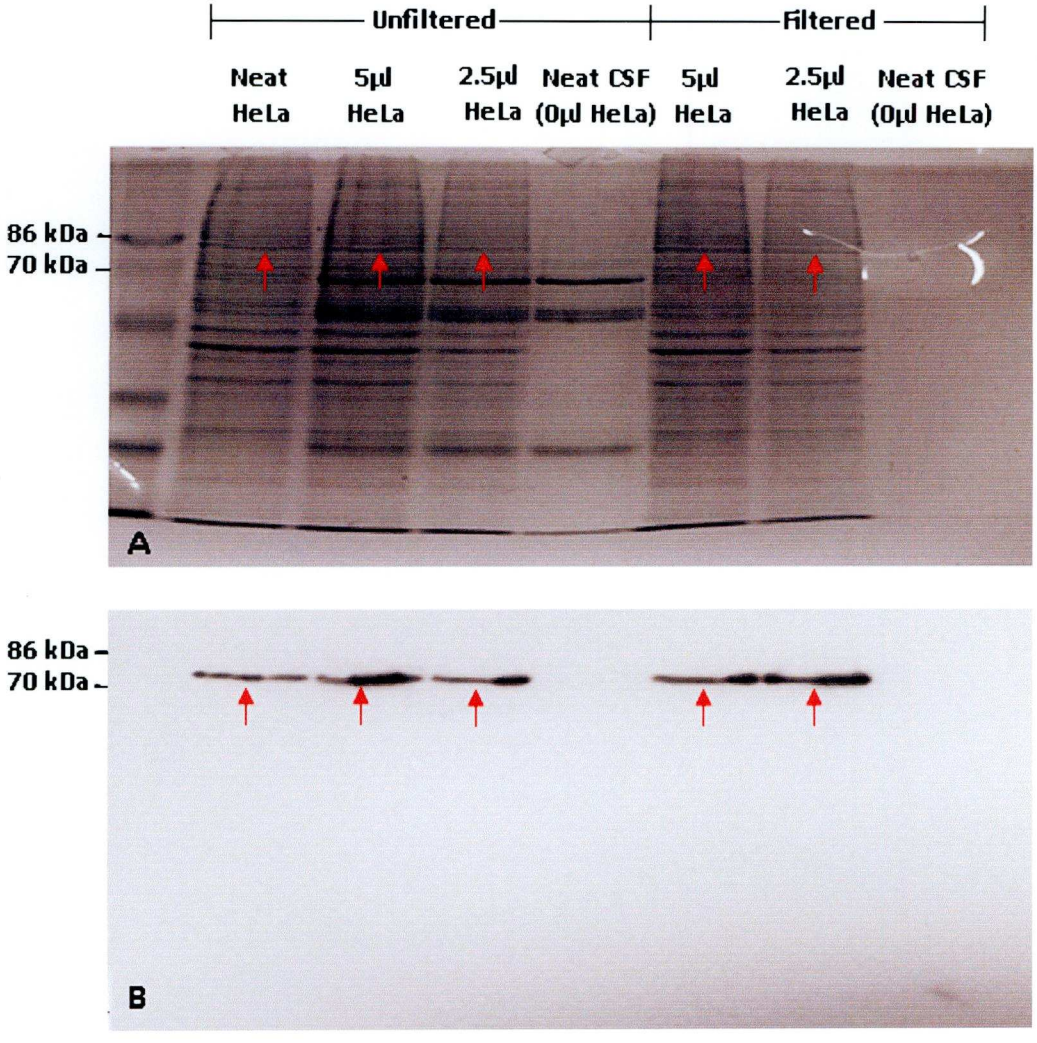

Figure 5.3 Filtration experiment for AIF. ***A*** *Coomassie stained SDS PAGE of the filtered and unfiltered CSF samples spiked with differing concentrations of the HeLa cell lysate. The AIF protein is indicated by a red arrow.* ***B*** *Western blot of the filtered and unfiltered CSF spiked with differing concentrations of the HeLa cell lysate. There appeared to be no confounding effect on the detection of AIF.*

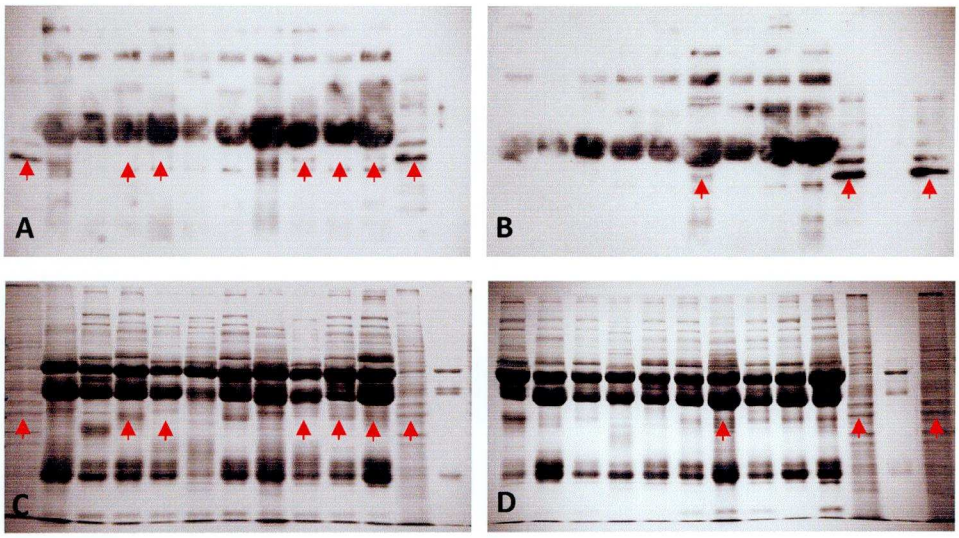

Figure 5.4. Western blot analysis of CKBB. ***A*** *Western blot of CKBB in non-survivors.* ***B*** *Western blot of CKBB in survivors.* ***C*** *SDS PAGE of non-survivor CSF samples.* ***D*** *SDS PAGE of survivor CSF samples. The suspected CKBB protein band is indicated by red arrows. The bands matched in the SDS PAGE and Western blot were excised from the gel and subject to in-gel tryptic digest followed by mass spectrometry. This confirmed the presence of CKBB.*

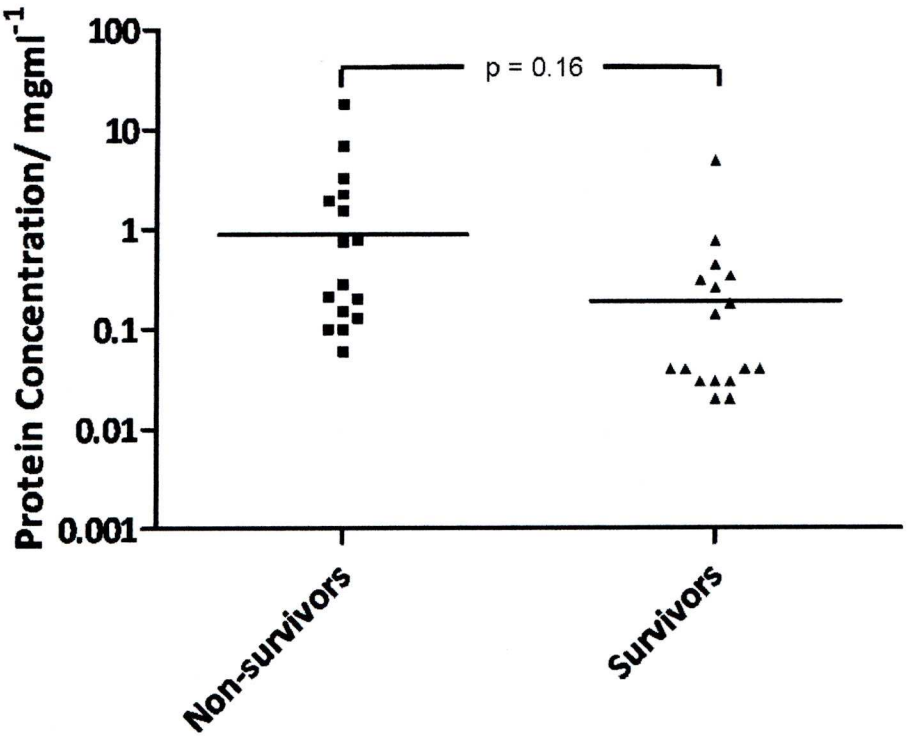

Figure 5.5 CKBB levels in CSF. *There was no statistical difference between the levels of CKBB in non-survivors and survivors (p = 0.16). However the level of CKBB was higher in non-survivors (average of 0.89 mgml^{-1}) than the amount of CKBB in survivors (average of 0.19 mgml^{-1}). CKBB was not found in normal CSF.*

Mass Spectrometry Confirmation of CKBB

The positive control marker was HeLa cell lysate spiked in normal CSF. In the Western blot CKBB was expressed at region of 43 kDa consistent with the molecular weight of CKBB (43 kDa). Despite a band appearing which was at a level common to the positive marker, there was significant cross-reactivity to cast doubt on the band observed being CKBB as shown in figure 5.4. Thus the band in each CSF sample was excised and sequenced using mass spectrometry. The protein was confirmed as CKBB through both MALDI-TOF and LC-MS/MS analysis with Mascot.

5.3.5 Expression of Proteins of CSF Origin

CSF Western Blot for Retinoic Acid X Receptor Gamma (RXRG)

In the previous 2D PAGE analysis, RXR gamma was identified as being downregulated 2-fold in non-survivors. In this Western blot analysis there was a significant difference observed between the mean concentration of RXR gamma in the non-survivor CSF samples (mean 0.69 $mgml^{-1}$) and the mean concentration in survivors CSF samples (mean 1.38 $mgml^{-1}$) used in the initial 2D PAGE analysis. A 2-fold downregulation in non-survivors was observed. This was identical to the fold difference observed in the initial 2D PAGE analysis.

In the analysis of RXR gamma in a larger sample size, RXR gamma was not found in normal CSF. However RXR Gamma was found in 73% of non-survivor CSF samples (mean 0.50 $mgml^{-1}$) and 83% of survivor CSF samples (mean 0.36 $mgml^{-1}$). Although the RXRG concentration was higher in non-survivors, there was no statistically significant difference between non-survivors and survivors ($p = 0.48$) as shown in figure 5.6.

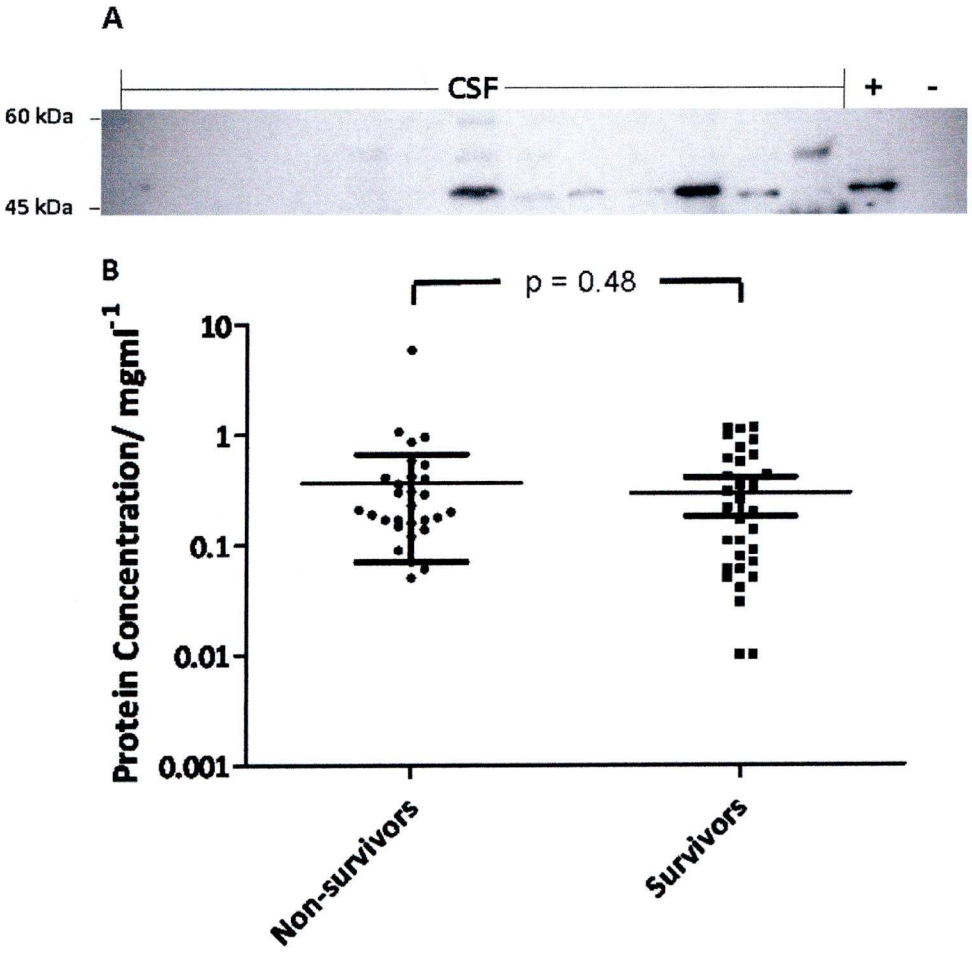

Figure 5.6 Retinoic acid X receptor gamma levels in CSF. ***A*** *Western blot of CSF samples with Retinoic acid X receptor gamma detection.* ***B*** *RXR gamma was not detected in normal CSF. There was no statistically significant difference between non-survivors and survivors (p = 0.48). However the amount of RXRG in non-survivor CSF samples (average = 0.50 mgml^{-1}) was higher than that in survivor CSF samples (average = 0.36 mgml^{-1}).*

CSF Western Blot for Zinc Finger Protein 179

In the 2D PAGE analysis, zinc finger protein 179 was previously identified as being upregulated 2-fold in non-survivors. In this Western blot analysis there was a significant difference observed between the mean concentration of zinc finger protein 179 in the non-survivor CSF samples (mean 0.22 $mgml^{-1}$) and the mean concentration in survivors CSF samples (mean 0.06 $mgml^{-1}$) used in the initial 2D PAGE analysis. A 2-fold upregulation of this protein in non-survivors was observed. This fold difference was identical to the fold difference observed in the initial 2D PAGE analysis.

In the analysis of Zinc finger protein 179 in a larger sample size, zinc finger protein 179 was not found in normal CSF however it was found in 98% of non-survivor CSF samples (average = 0.52 $mgml^{-1}$) and 70% of survivor CSF samples (mean 0.41 $mgml^{-1}$). Although its concentration was higher in non-survivors, there was no statistically significant difference between non-survivors and survivors ($p = 0.59$) as shown in figure 5.7.

CSF Western Blot for Chitotriosidase

Chitotriosidase was previously identified as being upregulated 5-fold in non-survivors. In this Western blot analysis there was a significant difference observed between the mean concentration of chitotriosidase in the non-survivor CSF samples (mean 1.19 $mgml^{-1}$) and the mean concentration in survivors CSF samples (mean 0.23 $mgml^{-1}$) used in the initial pilot proteomics analysis. A 5-fold upregulation of chitotriosidase was observed in non-survivors. This fold difference observed was identical to the fold difference observed in the initial 2D PAGE analysis.

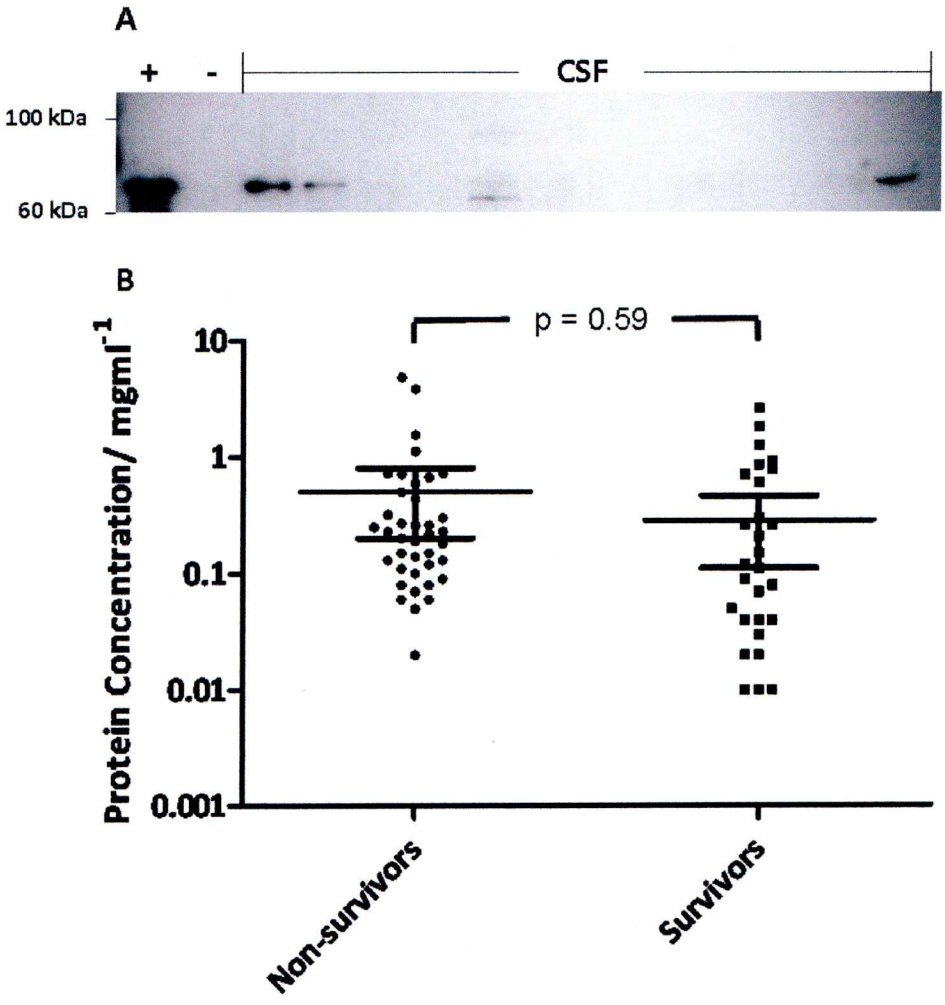

Figure 5.7 Zinc finger protein 179 levels in CSF. *In this analysis zinc finger protein 179 was not found in normal CSF. Zinc finger protein was found in higher amounts in non-survivor CSF samples (average = 0.52 mgml^{-1}) than survivor CSF samples (average = 0.41 mgml^{-1}). However, there was no statistically significant difference between non-survivors and survivors (p = 0.59).*

In the analysis of chitotriosidase in a larger sample size chitotriosidase was not found in normal CSF. Chitotriosidase was found in 50% of non-survivor CSF samples (mean 0.88 $mgml^{-1}$) and 75% of survivor CSF samples (mean 0.47 $mgml^{-1}$). Although the chitotriosidase concentration was higher in non-survivors, there was no statistically significant difference between non-survivors and survivors ($p = 0.09$) as shown in figure 5.8.

5.3.6 Expression of Proteins of Plasma Origin

CSF Western Blot for β-2-glycoprotein

In the initial 2D PAGE analysis, beta-2-glycoprotein was previously identified as being upregulated 3-fold in non-survivors in chapter 4. In this Western blot analysis there was no significant difference observed between the mean concentration of beta-2-glycoprotein in the non-survivor CSF samples (mean 0.97 $mgml^{-1}$) and the mean concentration in survivors CSF samples (mean 0.86 $mgml^{-1}$) used in the initial 2D PAGE analysis. Although there was no significant difference observed, the trend of beta-2-glycoprotein being higher in non-survivors was similar to the expression observed in the initial pilot proteomics analysis.

When Beta-2-glycoprotein was analysed in the larger CSF collection, beta-2-glycoprotein was found in normal CSF (mean 0.13 $mgml^{-1}$), non-survivor CSF samples (mean 0.83 $mgml^{-1}$) and survivor CSF samples (mean 0.69 $mgml^{-1}$) as shown in figure 5.9. Although the mean concentration of beta-2-glycoprotein was higher in non-survivors than survivors, there was no statistically significant difference between the groups ($p = 0.10$). There was however a significant difference between the beta-2-glycoprotein levels in clinically diagnosed pneumococcal meningitis CSF in comparison to normal CSF ($p < 0.0001$). There was no statistically different expression of beta-2-glycoprotein between non-survivors and survivors. The mean expression

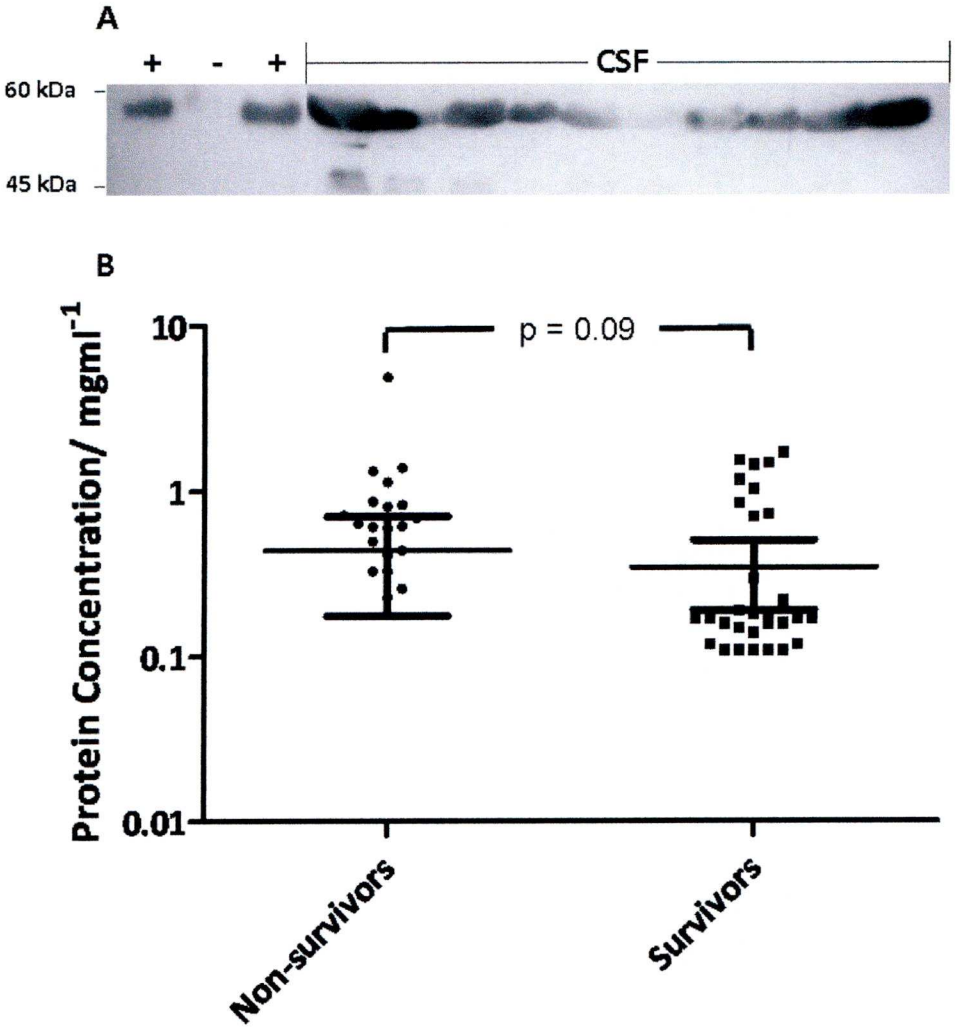

Figure 5.8 Chitotriosidase levels in CSF. *Chitotriosidase was not found in normal CSF. Chitotriosidase was found in higher amounts in non-survivor CSF samples (average = 0.88 mgml^{-1}) than survivor CSF samples (average = 0.47 mgml^{-1}). There was no statistically significant difference between non-survivors and survivors (p = 0.09).*

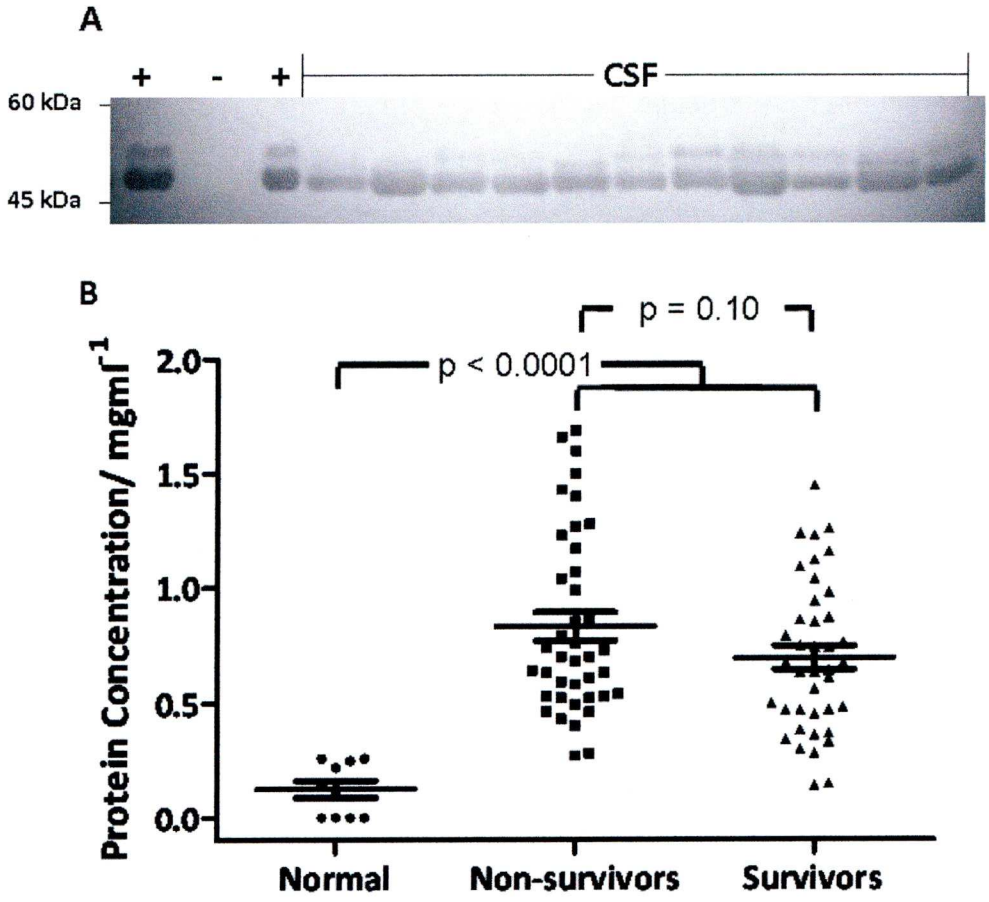

Figure 5.9 Beta-2-glycoprotein levels in CSF. *Beta-2-glycoprotein was found in normal CSF (average = 0.13 mgml^{-1}). Beta-2-glycoprotein was found in non-survivor CSF samples (average = 0.83 mgml^{-1}) and survivor CSF samples (average = 0.69 mgml^{-1}). There was no statistically significant difference between non-survivors and survivors (p = 0.10). There was a significant difference between meningitis CSF compared to normal CSF (p < 0.0001).*

levels of beta-2-glycoprotein were higher in non-survivors indicating a trend which followed the expression identified by proteomics.

CSF Western Blot for Complement C3 (C3)

In the pilot 2D PAGE analysis, C3 was previously identified as being downregulated 4-fold in non-survivors. In this analysis there was a significant difference observed between the mean concentration of complement C3 in the non-survivor CSF samples (mean 0.04 $mgml^{-1}$) and the mean concentration in survivors CSF samples (mean 0.21 $mgml^{-1}$) used in the initial pilot proteomics analysis. C3 was found to have a 5-fold downregulation in non-survivors. This fold difference observed was identical to that identified in the initial 2D PAGE analysis.

In the analysis of Complement C3 in the larger CSF sample collection, complement C3 was not found in normal CSF. However C3 was found in non-survivor CSF samples (mean 0.04 $mgml^{-1}$) and survivor CSF samples (mean 0.21 $mgml^{-1}$). There was a statistically significant difference between non-survivors and survivors ($p < 0.0001$) as shown in figure 5.10. Non-survivor CSF had 5-fold less C3 compared to survivors indicating a high dependence on C3 during pneumococcal meningitis. This increased level of C3 in survivors could indicate that C3 is not present in CSF of non-survivors or it is being rapidly used in non-survivors. This level of C3 correlated with the levels identified from the proteomic analysis.

CSF Western Blot for Transferrin

Transferrin was previously identified as being downregulated 5-fold in non-survivors. When the samples used in the initial 2D PAGE analysis was analysed using Western blot it was observed the there was a 2-fold reduction in the mean concentration of transferrin in non-survivors

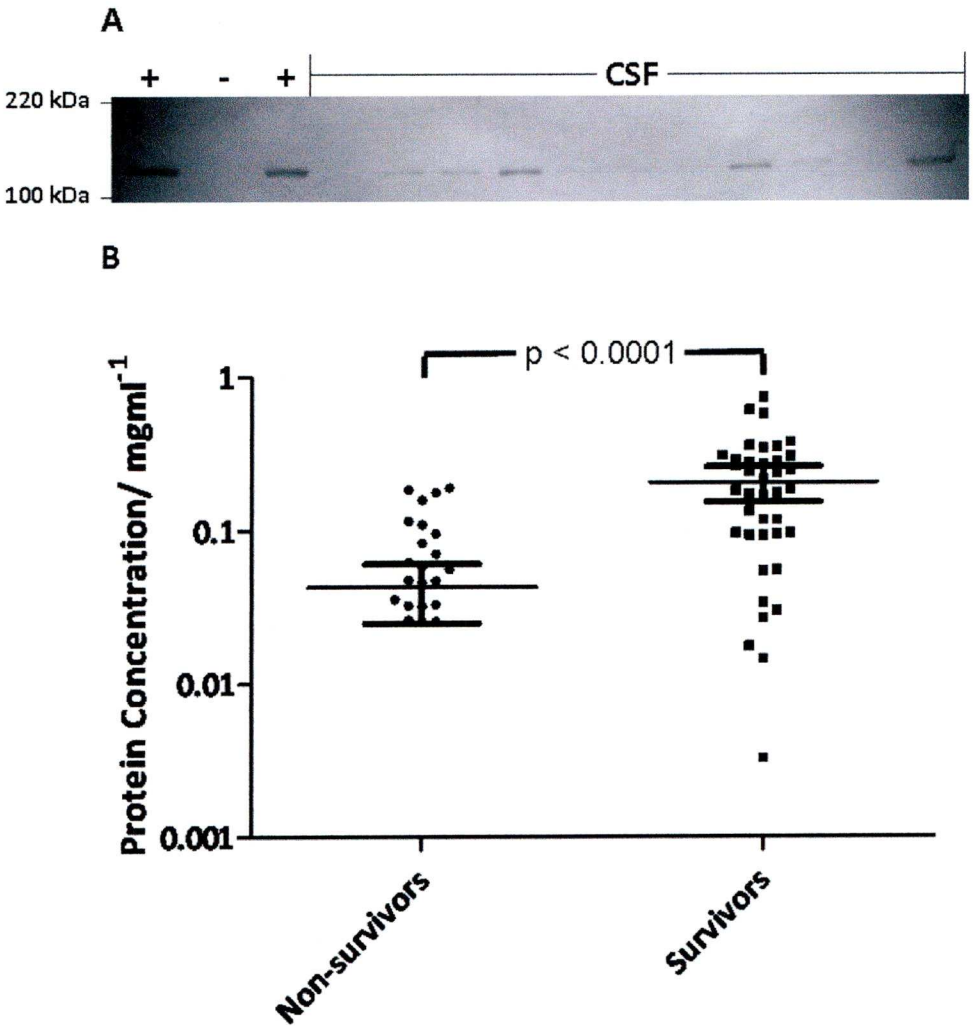

Figure 5.10 Complement C3 levels in CSF. *C3 was not found in normal CSF. C3 was found in non-survivor CSF samples (average = 0.04 mgml^{-1}) and survivor CSF samples (average = 0.21 mgml^{-1}). There was a statistically significant difference between non-survivors and survivors ($p < 0.0001$). Non-survivor CSF had 5-fold less C3 compared to survivors.*

(mean 0.72 $mgml^{-1}$) compared to survivors (mean 1.42 $mgml^{-1}$). This was comparable to the mean concentration observed in the pilot 2D PAGE analysis.

When transferrin was analysed in the larger sample size, transferrin was found in normal CSF (mean 0.08 $mgml^{-1}$), non-survivor CSF samples (mean 1.25 $mgml^{-1}$) and survivor CSF samples (mean 0.67 $mgml^{-1}$). There was a statistically significant difference between non-survivors and survivors ($p < 0.0004$) as the expression of transferrin in non-survivors was 2-fold higher than in survivors as shown in figure 5.11. There was also statistically significant difference between meningitis CSF and normal CSF ($p < 0.0001$).

5.4 Discussion

In this chapter the technique of Western blot was used to validate and identify protein targets in CSF. These proteins targets included proteins associated with cell death and proteins identified in previous chapters.

5.4.1 Analysis of Proteins Associated with Cell Death

The data obtained from the analysis of apoptosis proteins indicate that since caspase 3 and AIF are not present in this CSF collection there was no leakage of cell death material into CSF. This was surprising considering the level of data in animal models which suggest these proteins would be identified in CSF (33;189). The sensitivity of detection was increased by the use of polyclonal antibodies directed against caspase 3 and AIF at optimum levels of concentration and exposure to the CSF proteins.

The limit of detection was determined by serial dilution of the positive control. Both AIF and caspase 3 were detectable in the positive

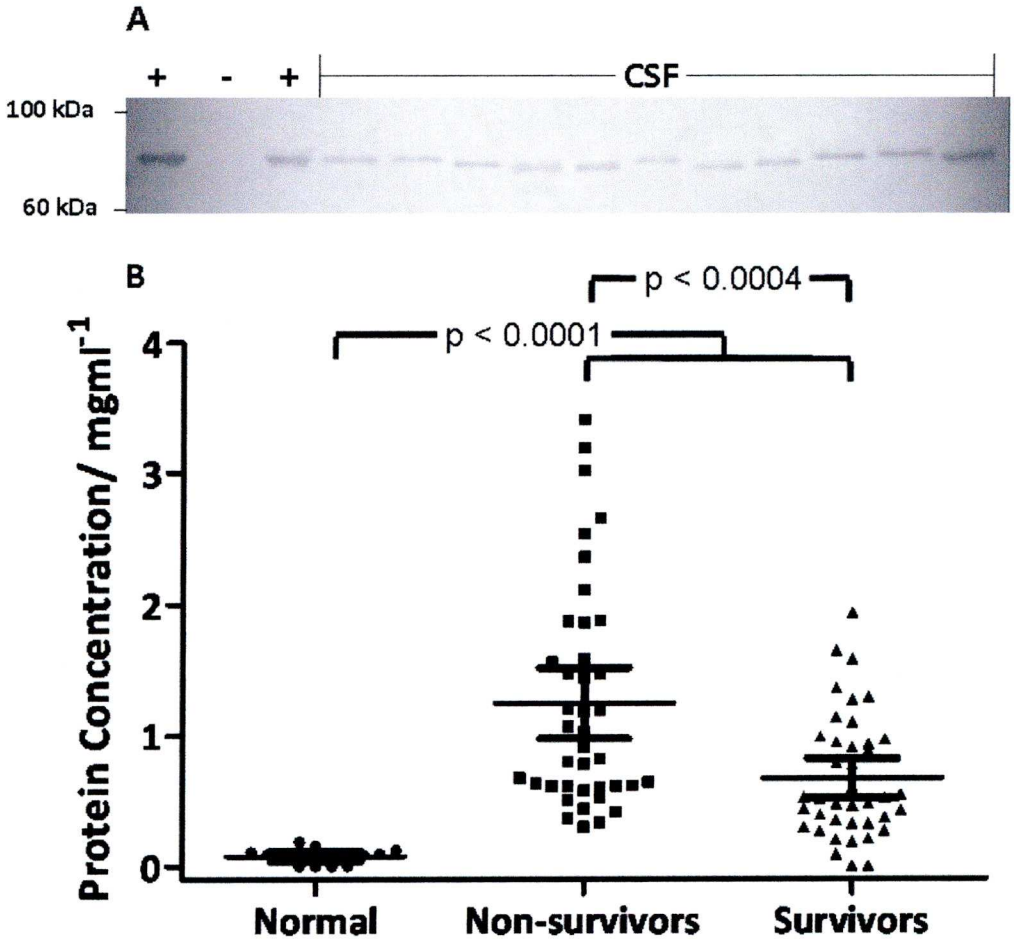

Figure 5.11 Transferrin levels in CSF. *Transferrin was found in some normal CSF (mean = 0.08 mgml^{-1}). Transferrin was found in non-survivor CSF samples (mean = 1.25 mgml^{-1}) and survivor CSF samples (mean = 0.67 mgml^{-1}). There was a statistically significant difference between non-survivors and survivors ($p < 0.0004$). Non-survivor CSF had 2-fold higher Transferrin compared to survivors. There was a statistically significant difference between meningitis CSF and normal CSF ($p < 0.0001$).*

cell lysate at protein concentrations as low as 100 $ng\mu l^{-1}$. The visualisation of the proteins in CSF upon spiking normal CSF with the control lysate would suggest that the proteins are not being confounded by larger proteins in CSF. It was possible that small foci of apoptosis did not result in a large increase of caspase 3 or AIF and this could be confirmed with suitable post mortem material.

There are alternative explanations for the lack of measurable apoptotic proteins. An increase in metabolic proteins was previously identified in chapter 4. Thus an increased metabolic rate may have led to an increase in AIF and caspase 3, undergoing rapid proteosomal degradation. These degradation pathways may occur via an ubiquitination process such as that mediated by X-linked inhibitor of apoptosis (XIAP) utilizing E3 ubiquitin ligase activity. This enables apoptosis inhibitors to catalyse ubiquination of itself, caspase-3, caspase 7 and AIF (190).

Alternatively other cell death pathways may be associated with pneumococcal meningitis in humans. The existence of multiple programs of cell death is strongly supported by the vast amount of information disclosed in recent years. Such pathways may include matrix metalloproteinases (MMPs) such as MMP9 and reactive oxygen species (ROS) which contribute to brain damage (191;192). Other pathways for apoptosis have also been described including; antiribosomal-P autoantibodies (193), brain-derived neurotrophic factor (BDNF) which blocks activation of caspase-3, reduces translocation of apoptosis-inducing factor (AIF), attenuates excitotoxicity of glutamate and increases antioxidant enzyme activities (34). Also Calpains (cysteine proteases activated by calcium during apoptotic processes) are known to activate proteins such caspase-12 after Ca^{2+} overload (194). Caspase-12 has been linked to neuronal degeneration in neurotoxicity animal models (195). Finally, STATs are a family of latent cytoplasmic proteins that are

involved in transmitting extracellular signals to the nucleus. Among the STATs, STAT3 has been shown to be involved in apoptosis (196).

In the meantime, however, the study has implications regarding treatment with caspase inhibitors and citicoline. These cannot, on the basis of these data, be expected to improve outcome in patients with pneumococcal meningitis.

Creatine kinase is a cytoplasmic protein released during tissue necrosis and CK-BB is the brain specific isotype of the protein. The presence of CKBB in CSF was an indicator that there was a low level of cortical necrosis in the CSF of patients (197). In this analysis the level of CKBB was expected to show a trend towards being higher in non-survivors based on other studies of CKBB in meningitis (44;198). In this analysis, however, the expression of CKBB was low which may have had an effect on the pairwise comparison. A more sensitive technique may be required to give a more accurate quantification and comparison. It is unclear whether oxidative modification may have had an effect on the outcome of the analysis (199). However there was no clear association of CKBB levels with clinical outcome.

In addition the expression of CKBB did not correlate with the expression of complement C3. This may indicate that the activities of these proteins were independent of each other.

5.4.2 Analysis of Proteins of CSF Origin

Three proteins that were found in chapter 4 were analysed as proteins of CSF origin. These included RXR gamma, zinc finger protein 179 and chitotriosidase. The concentrations of all three of these proteins varied significantly between patient samples.

It was expected that chitotriosidase would have a high expression in clinically diagnosed pneumococcal meningitis CSF but the expression was very low or absent. This protein has been identified as being a marker of macrophage activity (200). The lack of statistical difference in the mean concentration of chitotriosidase between non-survivors and survivors would suggest that there is no clear association of this protein with macrophage activity and outcome.

RXR gamma and zinc finger protein 179 are associated with protein transcription. As they are located primarily in the nucleus their low expression may be due to an incomplete leakage of cell material into the CSF. However the similar levels of these proteins suggest similar levels of transcription in non-survivors and survivors (144;147).

All three proteins were expressed in higher quantities in non-survivors than survivors but were not statistically significant. This expression trend corresponded with the 2D gel expression identified in chapter 3 for zinc finger protein 179 and chitotriosidase. However RXR gamma was found to have an opposite expression to that identified in chapter 3. This difference in expression may be a result of errors from the 2D PAGE analysis or from the mass spectrometry data.

5.4.3. Analysis of Proteins of Plasma Origin

Three proteins identified in chapter 4 were selected as proteins of plasma origin. These three proteins included beta-2-glycoprotein, complement C3 and transferrin. Unexpectedly the three proteins had differing results. The mean level of beta-2-glycoprotein expressed was higher in non-survivors, but there was no statistically significant difference between non-survivors and survivors. However the mean levels of C3 were significantly lower in non-survivors (5-fold), whereas the mean level of transferrin was found to be significantly higher in non-

survivors (2-fold). The level of C3 found in 'normal' blood plasma ranges from 0.75 $mgml^{-1}$ to 2.55 $mgml^{-1}$. The level of transferrin found in blood plasma ranges from 2.04 $mgml^{-1}$ to 3.06 $mgml^{-1}$. Therefore the levels of both C3 and transferrin found in CSF were significantly lower than the levels found in normal plasma. In this analysis the expression trends of C3 and beta-2-glycoprotein corresponded to the 2D gel expression identified in chapter 3. However transferrin was found to have an opposite expression to that observed in chapter 3.

Beta-2-glycoprotein (implicated in processes such as coagulation and atherosclerosis) had a higher mean concentration in non-survivors. The similar levels between non-survivors and survivors of this protein, which is associated with the blood clotting cascade, may indicate any effect on blood clotting during the breakdown of the blood-brain barrier by beta-2-glycoprotein may not have an association with outcome (150).

The first evidence for a functional role of the complement system in limiting pneumococcal outgrowth within the CNS was described by Tuomanen et al (201). In rabbits depleted of C3 by cobra venom factor, intracisternal inoculation of *S. pneumoniae* resulted in higher bacterial titres than in complement-sufficient control animals (202). Thus the lower levels of C3 in non-survivors may have been due to a greater need for C3 due to the severity of the illness. Angel et al, however, demonstrated that type 3 pneumococci express C3-degrading activity associated with the cell wall (67;203).

In addition pneumolysin can activate the classical complement pathway. High levels of pneumolysin in CSF may allow pneumococci to evade opsonophagocytosis by consuming the limited supply of complement factors (25).

Transferrin is a blood iron transport protein. The upregulation of this protein in non-survivors was most likely acting as an indicator of the degree to which the blood-brain barrier has leaked. The greater concentration of transferrin identified in non-survivors may be associated with blood-brain barrier damage. This blood brain barrier damage may in turn be a contributing factor to death from pneumococcal meningitis. Alternatively transferrin is commonly referred to as a negative acute phase protein. This is because transferrin levels tend to reduce during inflammation (204). Therefore an alternative theoretical explanation could be that survivors have an inflammatory process which is different to non-survivors. This unknown inflammatory process may be having an effect on outcome.

5.4.4. Western Blot as a Validation Tool

Although Western blotting is often considered qualitative, it also can be advantageous as a quantitative method. Quantitative Western blotting can be used for biological samples where no ELISA is available. Quantitative Western blotting could also be applied in situations where certain components in a biological sample may interfere with an ELISA. Often antibodies directed against one protein may interact with equal specificity with another closely related protein. In such situations an ELISA may generate false positives or overestimation of the target protein abundance. Because Western blotting involves gel electrophoresis, variations in molecular weight can be exploited to distinguish and quantify the target protein alone. Even though both Western blotting and ELISA rely upon immunodetection, they are different methods and as such are developed using specific antibodies and recombinant controls. Therefore, variation in absolute protein amounts may be caused by differences in the recombinant protein used for

controls, primary antibody specificity, and variations in protein interactions within each technique.

5.5 Conclusions

Western blot was able to confirm the presence of proteins identified previously in chapter 4 as well as confirm the presence of 1 of the 3 hypothesised proteins. Western blot however was only able to correlate the expression data identified from the initial proteomics analysis for the protein complement C3. The trend identified in the Western blot analysis appeared to correlate with the expression data identified in the initial proteomics analysis in 4 of the 6 proteins analysed. In addition to complement C3, the only other protein which appeared to give a significant difference between non-survivors and survivors was transferrin.

The lack of standard apoptosis markers would suggest that standard apoptotic pathways may be not occurring in this group of patients. However the presence of CKBB indicates cortical necrosis is present in some patients with pneumococcal meningitis.

Transferrin levels may indicate that blood-brain barrier damage is more severe in non-survivors. This may be having an effect on survival from meningitis. Thus future treatment may need to focus on protecting the blood-brain barrier from damage. This chapter has also shown that complement C3 may be playing a role in survival from pneumococcal meningitis. Future analysis of corresponding blood samples may provide confirmation of these results.

CHAPTER 6

HIGH LEVELS OF PNEUMOLYSIN AND NEURAMINIDASE A IN CSF OF PATIENTS WITH PNEUMOCOCCAL MENINGITIS

6.1 Introduction

The aim of this chapter was to determine if CSF samples obtained from patients clinically diagnosed with pneumococcal meningitis contain the pneumococcal proteins pneumolysin and/or neuraminidase A.

As described earlier in Chapter 1, pneumolysin (hemolysin or Ply) is a 53-kDa protein produced by all clinical isolates of *S. pneumoniae*. It is a pore forming toxin that requires activation by the addition of thiol-reducing agents (50). Ply can activate the complement pathway; however, immunoglobulin is required for Ply to bind to immunoglobulin G fragment crystallizable region ($IgGF_c$). Ply is able to induce leakage of solutes from erythrocytes, nucleated cells, liposomes, and conductance through planar lipid bilayers. It is presumed that the pores allow an influx of water leading to cell lysis, referred to as colloid-osmotic lysis.

Neuraminidases are a group of enzymes present in pneumococci which can cleave terminal sialic acid residues from a wide variety of glycolipids, glycoproteins, and oligosaccharides on cell surfaces or in body fluids (56). Coma and bacteraemia have been shown to occur significantly amongst patients with pneumococcal meningitis when the concentration of *N*-acetyl neuraminic acid in the cerebrospinal fluid is high. Pneumococci produce at least 2 distinct neuraminidases; NanA and NanB (205). NanA is the major neuraminidase and removes terminal sialic acid from glycoconjugates found on the surfaces of host cells (206). This is believed to expose cryptic receptors for the pneumococcus. There are several conflicting publications on the precise role of NanA in pneumococcal disease. However NanA-deficient pneumococci are significantly less able to colonise and persist in the nasopharynx and middle ear than NanA-sufficient wild-type pneumococci (57).

In this chapter four hypotheses were tested; (1) Pneumolysin is present in CSF, (2) NanA is present in CSF, (3) Poor outcome in pneumococcal meningitis is associated with the presence of either pneumolysin or NanA in CSF and (4) Ply and NanA expression levels change after antibiotic treatment.

6.2 Materials and Methods

All chemicals were purchased from Sigma Aldrich (Poole, UK), VWR (Lutterworth, UK) or Fisher (Loughborough, UK) unless otherwise stated. General solutions used for the analysis were made using double distilled water.

6.2.1 Patient Sample Information

The Western blot analysis was of CSF from non-survivors and survivors. After the initial lumbar puncture (t_0), a follow up lumbar puncture was carried after 48 hrs of treatment (t_{48}). The Western blot analysis was performed on CSF samples from the non-survivors t_0 samples (n = 5), non-survivors t_{48} samples (n = 5), the survivors t_0 samples (n = 24) and the survivors t_{48} samples (n = 24). The Western blots were conducted at the University of Leicester (Samia Hussain) in collaboration with the Liverpool School of Tropical Medicine. The low number of non-survivor CSF samples was due to the low number of non-survivors which survived to the second lumbar puncture. Normal CSF was obtained from patients who had presented with symptoms and were found to have no evidence of infection on lumbar puncture (n = 10). Information regarding the patients is given in table 6.1.

Table 6.1 Clinical Details of Patients Used in Pneumococcal Protein Analysis

	Cerebrospinal fluid sample		
	Control	***Non-survivors***	***Survivors***
	N = 10	N = 5	N = 25
Age - yrs mean (+/- SD)	27.8 (9.5)	30.8 (12.7)	31.7 (10.6)
Male sex - number (%)	3 (30)	1 (20)	12 (48)
GCS - mean (+/- SD)	9.4 (4.7)	10.0 (3.5)	11.8 (3.4)
Mean time to presentation (IQR, hrs)	55.2 (12 – 96)	58.4 (18 – 158)	78.1 (12 – 240)
Previous antimicrobials - number (%)	2 (20)	0 (100)	9 (36)
Microbiologic diagnosis - number (%)			
Proven bacterial			
S. pneumoniae	–	5 (100)	25 (100)
Not meningitis	10 (100)		
HIV Positive (% of those tested)	–	4 (80)	21 (84)
HIV Not known	10 (100)	1 (20)	4 (16)
Steroid Treatment – number (placebo)	–	2 (3)	17 (8)
Survival at day 10 (%)	–	5 (100)	25 (100)

The table above shows the patient details for the CSF samples analysed in the Western blot analysis for the presence of Ply and NanA. GCS Glasgow coma score, IQR interquartile range.

6.2.2 Antibodies and Control Markers

The antibodies for pneumolysin and NanA were supplied by the laboratory of Dr. Aras Kadioglu (University of Leicester). Work was carried out in collaboration between the Liverpool School of Tropical Medicine and the University of Leicester.

Antibodies

Pneumolysin and NanA antibodies were raised in mice at the University of Leicester. A summary of the antibodies used in this chapter are listed in Table 6.2.

Control Markers

Each of the proteins under investigation required a positive protein marker to confirm the identity of the protein under investigation. Purified pneumolysin and NanA was used as the positive control marker protein. The positive markers were recombinant proteins synthesised at the University of Leicester.

6.2.3 CSF Sample Preparation

CSF samples were stored at -20°C within an hour of sampling and at -80°C from 24 hrs until analysis.

6.2.4 Western Blot of CSF

Solutions for Western blotting were made up on the day of the experiment. The Western blot technique applied in this chapter is discussed in detail in chapter 2.

6.2.5 Statistics

Statistics were calculated using Prism software (v. 5.0, GraphPad, La Jolla, CA, USA) and R software (R project for statistical computing, free download from http://www.r-project.org/). Pairwise comparisons

Table 6.2 Antibodies Used in Pneumococcal Protein Analysis

Antibody phase	*Antibody*	*Antibody type*	*Specificity*	*Host*	*Detection*	*Supplier*
Primary	Pneumolysin	Polyclonal	*Streptococcus pneumoniae*	Rabbit	ECL	University of Leicester
	NanA	Polyclonal	*Streptococcus pneumoniae*	Rabbit	ECL	University of Leicester
Secondary	HRP conjugate secondary	IgG	Mouse	Goat	ECL	Sigma Aldrich

The table above shows the antibodies used in this analysis along with the corresponding conjugate used in the analysis.

were performed using an unpaired t-test with Welch's correction. Charts were plotted using Prism software.

6.3 Results

6.3.1 CSF Western Blot for Pneumolysin

The level of pneumolysin was measured in the CSF of non-survivors and survivors. There was no statistical difference between the levels of Ply in non-survivors and survivors ($p = 0.90$). There was no statistically significant difference between the levels of Ply in the survivor's t_0 samples (mean 0.08 $mgml^{-1}$) and survivor's t_{48} samples (mean 0.03 $mgml^{-1}$, $p = 0.08$). There was also no statistical difference between the non-survivors t_0 samples (mean 0.07 $mgml^{-1}$) and the non-survivors t_{48} samples (mean 0.07 $mgml^{-1}$, $p = 0.97$). No pneumolysin (Ply) was detected in normal CSF as shown in figure 6.1. Ply has been shown to induce cytotoxicity of the dentate gyrus at a concentration of 0.006 $mgml^{-1}$ (207). Spreer et al have discovered widespread toxicity of Ply at a concentration of between 85 $ngml^{-1}$ and 180 $ngml^{-1}$ (208). The expression mean discovered in these CSF samples was significantly higher than the concentrations seen in these previous studies.

6.3.2 CSF Western blot for NanA

The level of NanA was measured in the CSF samples of non-survivors and survivors. There was no statistical difference between the levels of NanA in non-survivors and survivors ($p = 0.31$). There was no statistically significant difference between the levels of NanA in the survivor's t_0 samples (mean 0.48 μgml^{-1}) and survivor's t_{48} samples (mean 0.33 μgml^{-1}, $p = 0.30$). There was also no statistical difference between the non-survivors t_0 samples (mean 0.3 μgml^{-1}) and the non-survivors t_{48} samples (mean 0.28 μgml^{-1}, $p = 0.91$). No NanA was detected in normal CSF as shown in figure 6.2.

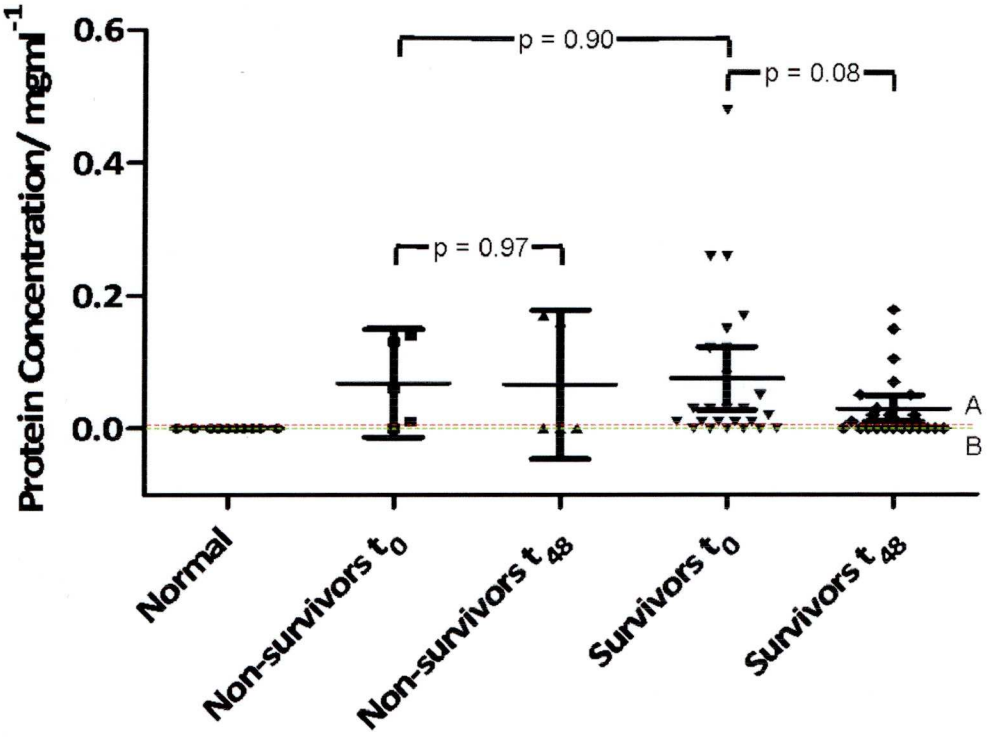

Figure 6.1 Comparison of pneumolysin in non-survivors and survivors. *There was no statistically significant difference between the levels of Ply in the survivor's t_0 samples (mean 0.08 $mgml^{-1}$) and survivor's t_{48} samples (mean 0.03 $mgml^{-1}$). There was also no statistical difference between the non-survivor's t_0 samples (mean 0.07 $mgml^{-1}$) or the non-survivor's t_{48} samples (mean 0.07 $mgml^{-1}$). Ply has been shown to induce cytotoxicity of the dentate gyrus at a concentration of 0.006 $mgml^{-1}$ represented by the red line **A**. (207). Spreer et al have discovered widespread toxicity of Ply at a concentration of between 85 $ngml^{-1}$ and 180 $ngml^{-1}$ represented by the green line **B** (208). The expression mean discovered in these CSF samples was significantly higher than the concentrations seen in previous studies.*

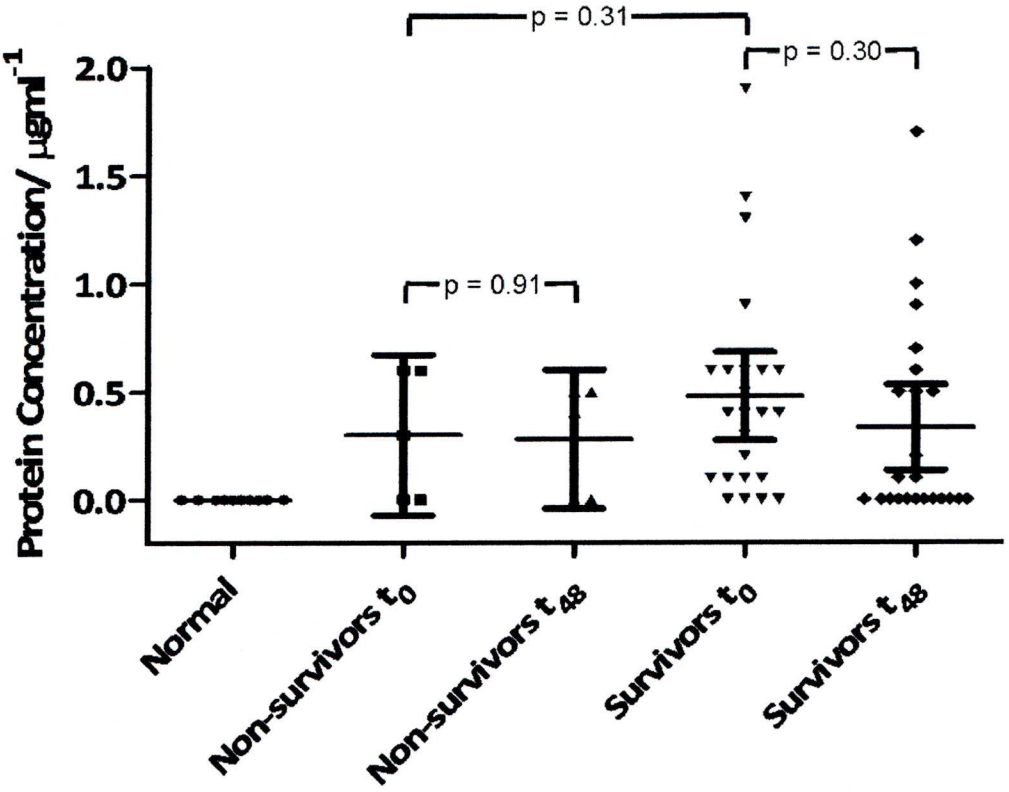

Figure 6.2 Comparison of NanA in non-survivors and survivors. *There was no statistically significant difference between the levels of NanA in survivor's t_0 samples (mean 0.48 μgml^{-1}) and survivor's t_{48} samples (mean 0.33 μgml^{-1}). There was also no statistical difference between the non-survivors t_0 samples (mean 0.3 μgml^{-1}) and the non-survivors t_{48} samples (mean 0.28 μgml^{-1}).*

6.4 Discussion

In this chapter we have shown that the proteins pneumolysin (Ply) and neuraminidase A (NanA) are present in CSF of patients clinically diagnosed with pneumococcal meningitis. Antibiotic therapy provided to patients successfully sterilised CSF.

6.4.1 Pneumolysin in CSF

There was no significant difference in the mean concentration of Ply in both non-survivor and survivor CSF samples at initial lumbar puncture. The mean concentration of Ply identified in these CSF samples was high compared to Ply concentrations identified in a previous study of pneumolysin in CSF from patients treated for meningitis (208).

After 48 hrs of treatment the level of Ply in non-survivors remained consistent with levels of Ply observed in the initial lumbar puncture. In the CSF samples, taken from patient survivors after 48 hrs of treatment, there was a reduction in the mean concentration of Ply observed in the CSF samples from the initial lumbar puncture. This was not a statistically significant reduction.

6.4.2 NanA in CSF

There was no significant difference in the mean concentration of NanA in both non-survivor and survivor CSF samples at initial lumbar puncture.

After 48 hrs of treatment the level of NanA in non-survivors remained consistent with levels of NanA observed in the initial lumbar puncture. In the CSF samples, taken from patient survivors after 48 hrs of treatment, there was a reduction in the mean concentration of NanA observed in the CSF samples from the initial lumbar puncture. This was not a statistically significant reduction.

6.4.3 Implications for Therapy

The implications of pneumococcal proteins persisting in CSF despite antibiotic therapy might suggest that there is a requirement for treatments aimed at neutralising toxins in order to improve outcome.

In previous chapters we have demonstrated that the host response is responsible for proteins with an association with survival. The presence of pneumococcal proteins in CSF, however, suggests that any future treatment may benefit from a focus on pneumococcal proteins. This may indicate that vaccine development targeting specific proteins of pneumococci may provide a greater protection than host specific target therapy.

In addition, in chapter 4 it was observed that there was a significant increase in the level of metabolic proteins present in CSF at the initial lumbar puncture. The observation that these pneumococcal proteins remain at a consistent level after 48 hrs would also suggest that these proteins are evading the standard metabolic pathways associated with protein degradation as a result of metabolism.

6.4.4 Effect of Pneumolysin on the Host Response

Although the pneumococcal proteins observed in this analysis may not be predictive of outcome, they are likely to be influencing the host response. Ply in particular has been significantly linked to neuronal cell death in animal models. In chapter 3 we were unable to find evidence of cell death markers in CSF. The presence of Ply could potentially be an explanation for the widespread levels of neuronal injuries despite no evidence of cell death markers. The mean expression of Ply was significantly higher than cytotoxicity levels discovered in previous studies of Ply.

The site of Ply production before detection in CSF remains unknown. It can be hypothesised that Ply is either produced externally of the blood brain barrier and the toxin aids entry of pneumococci across the blood brain barrier. Alternatively Ply may be produced in CSF by pneumococci leading to the host immune response and neuronal cell death.

6.4.5 Effect of Neuraminidase A on the Host Response

It is still not clear what specific role NanA has during pneumococcal infection. NanA was found to promote pneumococcal brain invasion in a recent murine model of meningitis (209). However it was not shown to be an important factor for invasion in another murine model of meningitis (210).

The lack of effect on outcome observed in this analysis strongly correlate with O'Toole and Stahl which states that pneumococcal neuraminidase played no significant role in the pathogenesis of neurologic dysfunction or death of dogs and, to the extent that findings in this model are applicable in the death of man (211).

The major effects of NanA are most likely associated with earlier processes of pneumococcal infection such as colonisation of the nasopharynx and with the spread of the pneumococcus from the nasopharynx to the lungs. Therefore the presence of NanA in CSF was most likely a result of the breakdown of the blood brain barrier. It is likely that NanA has an effect on the host inflammatory response. The extent of this effect remains to be determined.

6.5 Conclusions

Ply and NanA are present in CSF in high quantities. These proteins do not appear to be predictive of outcome from pneumococcal

meningitis. The mean concentration of Ply and NanA appear to remain constant in CSF from patients of non-survivors after 48 hrs of treatment. There is trend of reduction of the mean concentration in CSF of both Ply and NanA in survivors. But the mean concentration of both Ply and NanA remain high.

The implications of this are that despite surviving meningitis there is a risk of further complications at a later time point. Therefore host protein targets may not provide an adequate target for therapy. Thus there is a need to reduce the effect of these pneumococcal proteins. This will most likely be through development of protein specific vaccines.

None of these proteins were discovered in the previous proteomic analysis. This could potentially support the finding that there was no statistical difference in the level of expression of ply and NanA found in both non-survivors and survivors.

CHAPTER 7

FINAL DISCUSSION

7.1 Introduction

The information generated in this study implies that there are protein differences between CSF patient samples who are non-survivors and survivors. 2D PAGE research has illustrated that several 1000's of proteins are present in CSF. These proteins identified may yet form the basis of clinical point assays to assess the presence and activity of inflammation in meningitis, with a particular emphasis being placed on disease monitoring.

The technique of Western blot was used for both hypothesis testing and validation of mass spectrometry generated target proteins.

Western blot analysis was able to validate the incidence in CSF of the six proteins selected from the pilot proteomics analysis. Only complement C3 had a Western blot expression profile which correlated with the protein expression data observed in the 2D gel comparison analysis. However 4 of these proteins had an expression trend in line with the expression data from the 2D gel analysis.

Transferrin and complement C3 showed statistically different levels between non-survivors and survivors. Any association of transferrin and complement C3 with survival may allow new alternative treatment strategies for treating meningitis. The breakdown of the blood brain barrier appears to allow a large influx of proteins into the CNS which has a significant impact on the host inflammatory response. Thus indicating that protecting the blood brain barrier may improve outcome.

Cell death pathways found in animal models do not appear to correlate with the human forms of meningitis. Evidence of the classical apoptosis pathway was absent in CSF suggesting that treatment with apoptosis inhibitors will not have any impact as a treatment strategy. This

data from Western blot has been unable to support the use of citicoline and other methods of treatment. New hypotheses however have been generated leading to new studies which may suggest future treatment targets.

The presence of CKBB in CSF may indicate that neuronal necrosis occurs in meningitis at low levels. The low levels of CKBB suggest that necrosis may not be a major contributing factor to outcome.

The significant quantities of pneumolysin and NanA in CSF and the constant levels after 48 hrs of treatment indicate that therapy should be developed to minimise the efficacy of these pneumococcal proteins. This data is evidence that protein specific vaccines may prevent further complications from meningitis.

7.2 Differences between 2D PAGE and Western Blot Expression

Sample variability may account for differences observed in the expression data from proteomics and Western blot. Variability may be due to technical "procedural noise" or biological "genetic" variation. Understanding the degree of variation present in an experiment is central to all quantitative proteomic investigations. The degree of technical variation inherent to the 2D gel analysis was assessed using two-methods. Simple, pair-wise comparisons of spot volumes from two replicate 2D gels yielded a correlation coefficient (R^2), while for numerous repeat 2D gels the coefficient of variation (CV) (SD/ mean x 100) for all spot volumes on a gel was determined. Biological variation was measured using correlation coefficient (R^2) and the coefficient of variation (CV) (SD/ mean x 100) for all spot volumes on a gel from experiment- to-experiment repeats (inter-experimental replicates). The CV data for both technical and biological variation was high in both non-survivors and

survivors. There were extensive protein spots detected that overlap between survivors and non-survivors which highlights the complexity of the pathology of meningitis. This will have caused the coefficient of variation of the protein spots between samples to be high, which is a possible explanation for the lack of concordance observed between Western blot and 2D PAGE expression. As a result it is probable that this data underestimated the numbers of small proteins which differ between the groups due to technical limitations of 2D PAGE. Therefore an alternative approach may have been to fractionate the CSF and run 2D PAGE of the individual fractions. However the volume of CSF which can be collected from a lumbar puncture is low and this would have been impractical to carry out as a result. The results of this initial analysis suggest that improvements to the initial separating method will improve the level of detection of low abundant proteins in CSF.

There were also technical limitations of the Western blot technique applied. Western blot analysis is limited by the sensitivity of antibody detection. The data generated was semi-quantitative and only extreme differences in protein expression levels were discernable. Quantitative methods of mass spectrometry such as Isotope coded affinity tagging (ICAT) and iTRAQ mass spectrometry may provide an alternative to the use of Western blot.

7.3 Absence of Classical Inflammatory Markers

7.3.1 Classical Inflammatory Proteins

Previous protein analysis in meningitis and pneumococcal disease has often focused on the analysis of proinflammatory proteins including cytokines, chemokines and other inflammatory proteins. In particular previous analysis has identified the proteins: IL-1β, IL-6, IL-10, MIF, and TNF-α, CXC chemokines (ENA-78, GRO, IL-8, IP-10, and NAP-2), CC

chemokines (MCP-1, MCP-2, and MIP-3α and the growth factors HGF, IGFBP-1, and MCSF as being significant in the progression of pneumococcal disease towards fatal outcome from meningitis (133). Similarly, work on CSF from patients with malaria have previously identified similar proteins: IP-10, IL-1ra, IL-8, IP-10, PDGFbb, MIP-1β, Fas-L, sTNFR1 and sTNF-R2 in Ghanian children (100). None of these proteins were identified in our list of protein differences between non-survivors and survivors.

7.3.2 Possible Explanation for the Absence of Inflammatory Proteins

These proteins have previously been identified in a cytokine analysis of the CSF samples used in this study. Therefore the absence of these proteins is unlikely to have a biological cause and is more likely to be due to technical reasons. Long term storage of samples and inadequate collection methods required for future proteomics analysis could potentially account for the absence of cytokine data. Alternatively the differences in expression of these proteins could be below the limits of detection for 2D PAGE spot analysis. It has been demonstrated that a single spot on a 2D gel of a yeast proteome consists of a minimum of 6 proteins. In humans this number will be much higher. Therefore it is possible that these proteins were confounded by large quantities of other endogenous proteins present in CSF from patients clinically diagnosed with meningitis such as albumin and immunoglobulin. This may also have influenced the data generated by mass spectrometry. The final peptide spectra is normalised to the highest abundant peak present in a peptide mixture. Therefore more abundant peptide ion species present in a spot digest mixture at the time of mass spectrometry will have inhibited the final database search. Alternative approaches to global scale protein analysis such as 2-Dimensional LC-MS/MS may overcome this limitation.

7.4 Fixed-Time Point Analysis

There is potential for these techniques to be applied to various time points in the disease. As discussed, the CSF used in this study was collected from patients at two time points. CSF was collected by lumbar puncture from the patient after initial diagnosis and again after 48 hrs of antibiotic therapy. Protein levels constantly rise and fall. The time between the patient showing symptoms and presentation may have influenced the concentration of protein found in CSF at the initial lumbar puncture. Analysis at more time points would therefore provide greater accuracy in the analysis. However this would be difficult and unethical to obtain.

7.5 Mode of Death in Pneumococcal Meningitis

In this analysis proteins in CSF were analysed for an association with outcome. This analysis was limited by only using two phenotypes for comparison i.e. survival and death. The overall concluding analysis suggested there was no clear association with outcome of the proteins analysed in the larger CSF collection other than complement C3 and transferrin.

In many cases, the clinical outcome at the end of primary hospitalisation was not definitive and evolved in subsequent weeks and months. In the initial clinical trial study, the predefined primary outcome was mortality at 40 days from randomization by intention to- treat analysis. Secondary outcomes were time to death, combined disability and death as defined by the Glasgow Outcome Score (1, death; 2, vegetative state; 3, severe disability; 4, moderate disability; 5, mild or no disability) at day 40, hearing impairment at day 40, death at 10 days, and death at 6 months (2).

Therefore the conclusion of this study suggests a need to increase the phenotype groupings used in the comparison analysis. The phenotypes included in any future studies may include measured neurological sequelae including hearing deficiency, paresis, encephalopathy, vestibular syndrome, mental disease, severe headache, epilepsy and more severe sequelae including vegetative state apallic syndrome (212).

In other case studies of meningitis which have looked at outcome (213); the following variables were found to be associated with an increased risk of unfavourable outcome: age, predisposing infectious focus, internal comorbidity, > 48 hrs to treatment, coma (low GCS score), general compromise (high APACHE II score), hypotension, convulsions, high CSF protein, low glucose ratio, pneumococcal and Gram-negative aetiology, and non-meningococcal aetiology.

Independent predictors of unfavourable outcome: internal comorbidity, > 48 hrs to treatment, coma, hypotension, high CSF protein, low glucose ratio, and non-meningococcal aetiology (213).

7.6 Hypothetical Example of a Potential Treatment Strategy

The level of transferrin and complement C3 require quantification in the corresponding plasma samples of the CSF samples used in this study to confirm the origin of protein expression data observed. This work is ongoing in the laboratory.

A hypothetical example of how therapy may be developed using the transferrin data may involve providing treatment to improve the resistance of the blood brain barrier if transferrin data correlates with blood brain barrier damage. It may be possible to reduce mortality associated with pneumococcal meningitis by protecting the blood brain

barrier. An example of a treatment strategy could involve inhibiting initial blood brain barrier insults associated with apoptosis of endothelial cells. Therefore although anti-apoptotic drugs may not have any benefit in treating pneumococcal disease at the stage of meningitis as discussed in chapter 5, they may have a beneficial role in providing protection to the blood brain barrier. It must be noted, however, that blood brain barrier leakage may allow entry of critical defence molecules and cells such as IgG and neutrophils.

In another hypothetical example of how therapy may be designed from complement C3 data. Low levels of C3 may require an artificial method of increasing the levels of C3 to increase pathogenic resistance. One such method may involve the artificial use of estradiol which has been demonstrated to induce C3 gene expression (214).

7.7 Future Experiments

Initial protein separation methods are inhibited by the presence of large endogenous proteins. Therefore in order to detect low abundance proteins, alternative techniques are required. Such techniques include:

7.7.1 2D LC-MS/MS

To analyse a complex protein sample by LC-MS/MS, a so called "shotgun sequencing" approach is often used. The sample under analysis e.g. CSF is digested directly with a suitable protease, commonly trypsin, and the resulting peptides are separated by HPLC into fractions (the 1st dimension). These fractions can then be re-separated (the 2nd dimension) by HPLC and characterised by tandem MS. This effectively increases the detection of low abundance proteins. Proteins are identified by matching the MS fragmentation patterns with predicted information

from genomics or proteomics databases. More peptides can be loaded and comprehensively analysed with 2D LC-MS/MS (215).

7.7.2 Alternatives to Western Blot

iTRAQ

Isobaric tagging of peptides enables high-throughput proteomic analysis. iTRAQ reagents allow simultaneous identification and quantitation of proteins in four different samples using tandem mass spectrometry (MS) (216).

ICAT

Isotope-coded affinity tags (ICAT) is a method which involves labelling amino acids with chemical probes. These chemical probes consist of 3 general elements: a reactive group capable of labelling a defined amino acid side chain (e.g. iodoacetamide to modify cysteine residues), an isotopically coded linker, and a tag (e.g. biotin) for the affinity isolation of labelled proteins/peptides. For the quantitative comparison of two proteomes, one sample is labelled with the isotopically light (d0) probe and the other with the isotopically heavy (d8) version (217).

7.7.3 Fourier-Transform (FT) Mass Spectrometry

FT mass spectrometry allows increased sensitivity for the detection of low abundance proteins. There are two key FT mass spectrometers available:

LTQ Orbitrap

The linear trap quadrupole (LTQ) orbitrap is the upgrade to the ion trap system used in this analysis. The benefit of this system is for database searching and PTM assignment. The Orbitrap is able to identify many more proteins. High mass accuracy is achieved with the Orbitrap

mass analyser which has a direct benefit on the quality of protein identifications. Many more predicted ions are matched in the Orbitrap spectrum which significantly improves Mascot scores for these peptides (218).

FT Ion Cyclotron Resonance (ICR)

FT mass spectrometers are expensive, but they have a much improved signal-to-noise ratio (S/N), higher sensitivity and higher resolution than most other mass analysers (113). FT ICR mass spectrometers contain a type of ion-trap within which ions are allowed to circulate in defined orbits over extended periods of time. The behaviour of the ions is described by the ICR phenomenon (219).

7.7.4 Metabolomics/Metabonomics

Metabolomics is the systematic study of the unique chemical fingerprints that specific cellular processes leave behind with mass spectrometry. Metabonomics utilises NMR for the same analysis (220). The presence of large amounts of metabolic proteins would suggest that a metabolomic analysis of the CSF may provide new answers to differences in the physiology of CSF with pneumococcal meningitis.

7.8 Final Conclusion

Proteomics has not delivered the expected insights that might drive the next treatment for pneumococcal meningitis. However 3500 proteins were found to be different between CSF from patients with pneumococcal meningitis and CSF from 'normal' patients. This would indicate a high probability that there are more proteins to be validated and analysed in pneumococcal meningitis CSF as specific for the disease. In addition many of the proteins that have been discovered in this sample set have yet to be analysed further. Thus they may still provide new

information regarding the protein biology of CSF during pneumococcal meningitis.

This global scale analysis of proteins in CSF has a potential advantage over targeted measurements of proteins in CSF as analysis of single proteins is both rate-limiting and unlikely to reveal information regarding the collective protein associations found in pneumococcal meningitis. However until all technical limitations are overcome, targeted analysis of proteins using Western blot provides the most adequate method for CSF research. Therefore proteomics in biomarker discovery in CSF meningitis studies still has some way to go before it becomes a truly informative tool.

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Appendix A

CSF Examination

Day 1

1. Number the sample and enter the patient details in the CSF daybook.

2. Appearance (note the appearance of the CSF on front of form):

a) Clear and colourless

b) Hazy

c) Cloudy/ turbid

d) Bloodstained/ clotted

e) Frankly purulent

f) Xanthochromic

3. Samples should be treated as follows:

Appearance	Cell count	Protein	Glucose	Indian Ink	Gram	Differential	BA	CHOC	BHI
Clear	✓	✓	✓	✓*			✓	✓	
Cloudy	✓	✓	✓	✓*	✓	✓	✓	✓	✓
Bloodstained	✓	✓**	✓**	✓*	✓+	✓+	✓	✓	
Clotted (Blood)					✓		✓	✓	✓

* All adult samples and paediatric samples on request ** If not heavily bloodstained $^{+}$ If indicated by the cell count

4. Cell Count

a) Using a sterile pipette, fill a Modified Fuchs Rosenthal chamber with CSF

b) Allow the chamber to stand for a few min to allow any cells to settle.

c) Count the number of white and red blood cells present in 5 large squares (see Fig.1, below), under x 40 magnification

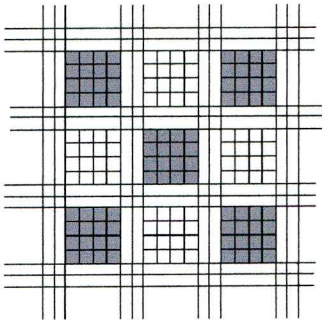


Fig 1. Modified Fuchs Rosenthal Counting Chamber

d) Note the number of white and red cells as:

i) No cells seen - report 0

ii) Cells seen - report (The number seen)/ mm^3 e.g. 240/ mm^3

e) If there are >40 cells per large square count a single square and multiply the answer by 5.

f) If there are >40 cells per small square count a single square and multiply the answer by 80.

g) If there are too many WBC cells to count accurately, dilute the CSF 1:10, 1:20, 1:50, or 1:100 (as appropriate) in saline. Adjust the count using the appropriate dilution factor.

h) If the sample is heavily bloodstained/ clotted report 'Not available' against the RBC count and prepare slides for Gram stain.

5. Protein and Glucose

Using a sterile pipette put a drop of CSF onto the protein and glucose sections of a urine dipstick. Report as indicated by the dipstick instructions.

NB: Heavily bloodstained/ clotted samples are should not be tested as the high RBC content interferes with interpretation of the strip.

6. White Cell Differential

a) To be carried out on all CSF samples where >20 WBC's are seen in the cell count.

b) Make a film direct from the CSF if cloudy, or from the spun deposit.

c) Allow to air dry or dry on the edge of a hot plate to avoid cell distortion.

d) Stain with Wright's stain (see Identification Tests for method)

e) Examine the slide under X100, oil immersion and count the number of polymorphs and lymphocytes present, expressing the result as a %.

7. Gram Stain

a) To be carried out on all CSF samples where >20 WBC or organisms are seen in the cell count.

b) Make a film either direct, if cloudy, from the CSF, or from the spun deposit.

c) Dry the film on a hot plate and Gram stain, (see Identification Tests for method).

d) Examine the slide under X100, oil immersion and report the presence of any organisms and quantify as below:

\+ 1- 10 organisms/ High Power Field (HPF)

++ 10- 100/ HPF

+++ >100/ HPF

8. Indian Ink Stain

a) Carried out routinely on adult CSF samples; on paediatric samples only on request.

b) Using a sterile pipette put a drop of CSF onto a clean slide.

c) Put a drop of Nigrosin stain onto the same slide.

d) Mix the two together and cover with a cover slip.

e) Examine the slide at x40 magnification.

f) If any capsulated yeast cells are seen, report as Positive and quantify as below:

\+ 1 per 10 fields

++ 1-10/ field

+++ >10/ field

NB: Report all positive microscopy results to the ward using the preliminary result sheets.

9. Ziehl Neilson (To be carried out if advised by the clinical microbiologist)

a) CSF Culture

i) Spin all CSF's at 3000rpm for 15min

ii) Decant the supernatant of samples requiring storage, into a sterile labelled cryotube tube and store at –20°C, (see list on wall in MRP Lab). If there is any doubt about which samples should be stored discuss with the senior microbiologist.

iii) Discard supernatant of negative samples into a discard jar containing disinfectant.

iv) Culture the deposit as indicated below:

	MEDIA							
	¼ CHOC (CO_2)	¼ BA (CO_2)	¼ SAB (O_2)	BHI (O_2)	ISC (CO_2)	¼ CLED (O_2)	ISB (CO_2)	ISO (O_2)
All samples	✓	✓						
gnr	✓	✓			✓*	✓		✓
gpdc		✓					✓	
gndc	✓	✓					✓	
Yeast	✓	✓	✓					
nos	✓	✓		✓				

gnr-Gram negative rod; gpdc- Gram positive diplococci; gndc- Gram negative diplococci; nos- No organisms seen

*Only paediatric samples

b) ISC, ISO, and ISB are whole plates used for antibiotic sensitivity testing (see below)

10. Direct sensitivity Testing

a) Direct antibiotic sensitivity testing should be carried out on all samples where ≥++ organisms are seen on the Gram stain.

b) Use the appropriate plate indicated in the table above.

c) Using a sterile pipette put a drop of CSF into the middle of the plate.

d) Spread the drop evenly over the surface of the plate, in 3 directions using a sterile cotton wool swab.

e) After ensuring that the plate is dry, apply the appropriate antibiotic sensitivity discs, see table below.

f) Incubate as indicated by BSAC SOP.

GNR- ISO (coliform)	GNR- ISC (? HI)	GNDC- ISB	GPC- ISB (? Strep)
Ampicillin 10	Ampicillin 2	Tetracycline 10	Tetracycline 10
Ceftriaxone 30	Tetracycline 10	Co-trimoxazole	Co-trimoxazole
Gentamicin 10	Gentamicin 10	Ceftriaxone 30	Erythromycin 5
Co-trimoxazole	Co-trimoxazole	Chloramphenicol 10	Chloramphenicol 10
Chloramphenicol 30	Chloramphenicol 10	Ciprofloxacin 1	Oxacillin 1
Ciprofloxacin 1	Ceftriaxone 30	Penicillin 1	Ceftriaxone 30

Day 2

1. Plates

a) All plates should be examined after 24 hours and any growth identified, see organism identification SOP.

b) If significant organism isolated carry out antibiotic sensitivity testing for the appropriate organism.

c) Re-incubate for a further 24 hrs if no growth occurs.

NB: Save all isolates as indicated in Sample storage SOP

Day 3

1. Plates

   a) Examine all re-incubated plates and identify any growth.

   b) If no growth and <20 WBC's report as: No growth after 48hrs.

2. BHI Broth

   a) Incubate the BHI broth at 37°C in CO_2 for 48hrs.

   b) If the direct culture plates show no growth after 48hrs, subculture the BHI. BHI samples should be sub cultured to ¼ CHOC and ¼ BA.

Day 4

1. Examine BHI subculture plates and identify any growth.

2. If no growth occurs re-incubate the plates for a further 24hrs.

Day 5

1. Examine BHI subculture plates and identify any growth.

2. If no growth report as: No growth after 4 days

Appendix B

Solutions Used

(1) Tris HCl was prepared using 6% Trizma base with the pH adjusted to 6.8.

(2) 1.5 M Tris base was prepared with pH adjusted to 8.8.

(3) PBS was prepared by dissolving 1 tablet of PBS in 200 ml distilled water.

(4) Tank buffer (composition: 3% w/v Tris base, 14.4% w/v glycine and 1% w/v SDS).

(5) CBB (composition: 0.125% (w/v) CBB G250, 10% (w/v) ammonium sulphate, 2% (v/v) orthophosphoric acid and 25% methanol).

(6) CBB destaining solution 1 (composition: 10% (v/v) acetic acid in 25% (v/v) methanol).

(7) CBB destaining solution 2 (composition: 25% (v/v) methanol).

(8) Fixing solution (composition: 5% (v/v) acetic acid, 40% (v/v) absolute ethanol).

(9) Silver nitrate solution (composition: 0.2% (w/v) silver nitrate and 0.000025% (v/v) formaldehyde).

(10) Silver developing solution (composition: 3% (w/v) sodium carbonate, 0.000025% (v/v) formaldehyde and 0.00005% (w/v) sodium thiosulphate).

(11) Silver stopping solution (composition: 5% (w/v) Trizma base and 2% (v/v) acetic acid).

(12) 2x sample buffer (composition: 25% (v/v) Tris-HCl pH 6.8, 40% (v/v) 10% SDS, 20% (v/v) glycerol, 20% (w/v) Bromophenol blue and 10% (v/v) 2-mercaptoethanol).

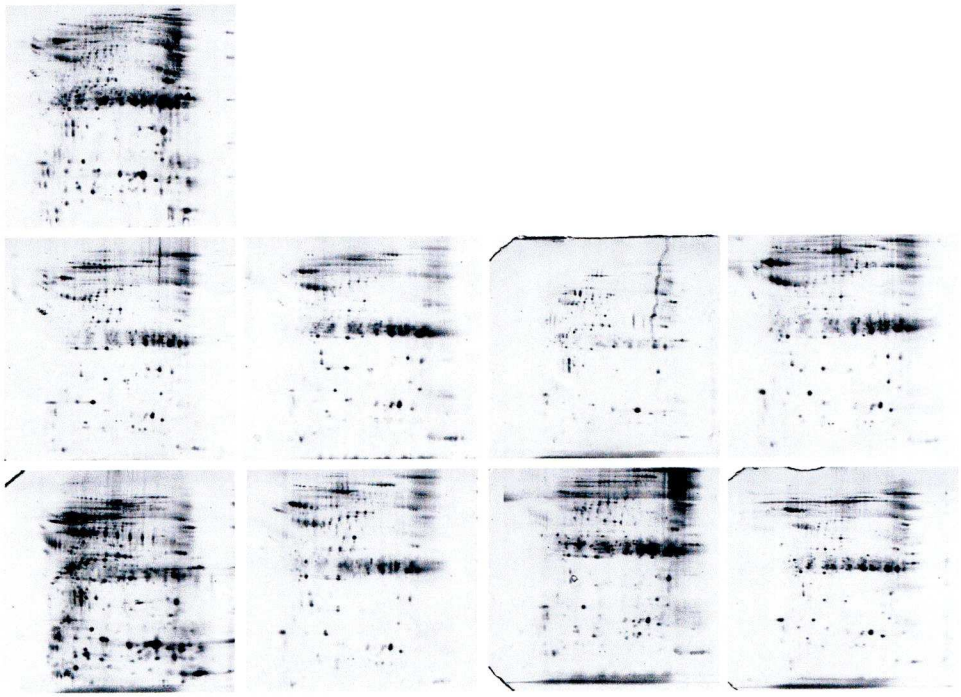
(13) Towbin transfer buffer (composition: 25mM Tris pH 8.3 consisting of 3% w/v Tris base, 14.4% w/v glycine (192mM) and 20% v/v methanol).

(14) TBS-T (composition: 0.12% w/v Tris base, 0.88% w/v Sodium Chloride and 0.1% v/v Tween-20 adjusted to pH 7.0).

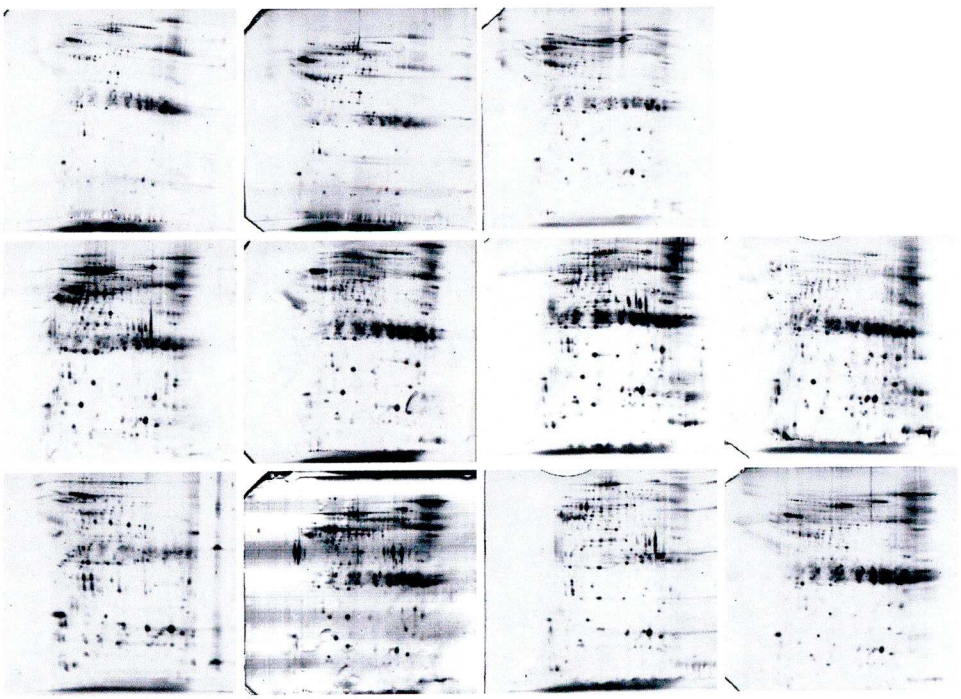
(15) DAB substrate (composition: 200 ml 1x PBS, 0.05% w/v diaminobenzadine tetrachloride and 0.03% v/v hydrogen peroxide).

Appendix C

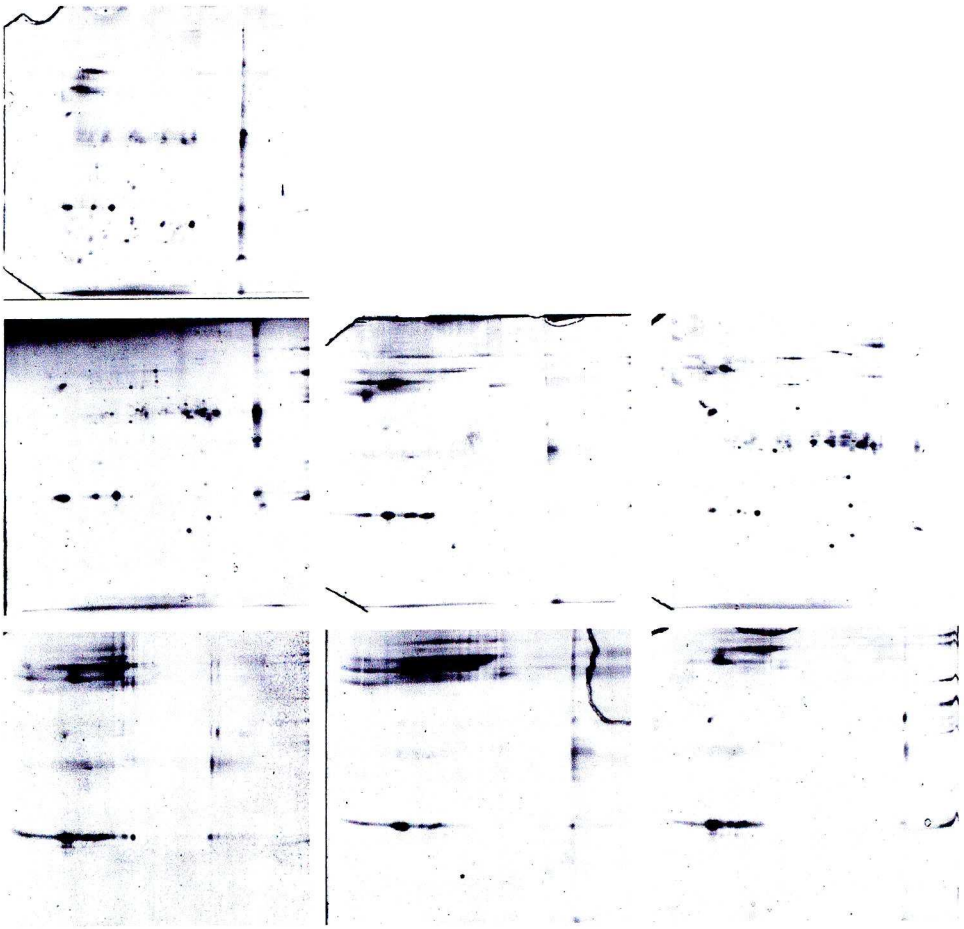
Survivor gels



Non-survivor gels

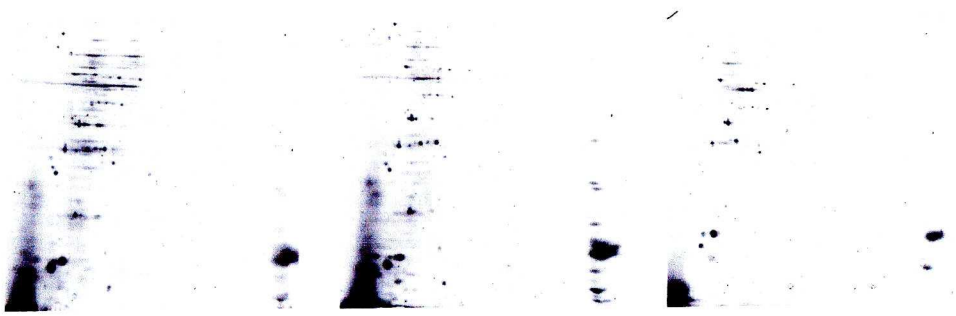


Normal Gels



Pneumococcal Gels

Sp1 **Sp6b** **Sp14**



Appendix D

Additional Information of MALDI Identified Downregulated Proteins

Protein name	*Mascot Score >55*	*Theoretical Mr/pI (kDa/pH)*	*Experimental Mr/pI (kDa/pH)*	*Accession Number*	*Sequence coverage (%)*
Phosphoglucomutase-like protein 5 (PGM5)	57	63, 6.81	60, 5.90	Q15124	13
Cancer associated gene 1 (CAGE1)	58	64, 6.51	58, 5.30	Q8TC20	23
Chain A, Solution Structure Of Domain 3 From Human Serum Albumin (ALBU)	56	23, 8.22	20, 6.00	P02768	34
T-complex protein 1 subunit zeta (TCPZ)	81	58, 6.23	66, 5.90	P40227	17
Brain-enriched guanylate kinase-associated protein (BEGIN)	56	65, 5.40	60, 5.80	Q9BUH8	12
Glyoxalase domain-containing protein 4 (GLOD4)	57	35, 5.40	59, 5.60	Q9HC38	21
Retinoic acid receptor RXR-gamma (RXRG)	58	51, 7.55	50, 5.50	P48443	15
Zinc finger protein 1 (ZIK1)	59	45, 9.53	43, 5.60	Q3SY53	25
Serine/Threonine phosphatase 2-alpha 65K regulatory chain (2AAA)	65	45, 6.13	43, 5.40	P30153	18
Serine/threonine/tyrosine-interacting-like protein 1 (STYL1)	61	25, 5.44	40, 5.40	Q9Y6J8	45
Nuclear localized factor 1 (NLF1)	57	40, 11.28	40, 4.70	Q8NCU7	21
Ankyrin repeat domain-containing protein 42 (ANR42)	59	44, 6.01	40, 4.70	Q8N9B4	17
Eukaryotic translation initiation factor 2, subunit 2 beta (EIF2S2)	63	38, 5.60	38, 5.20	Q6IBR8	25
Ras-related protein Rab-37 (RAB37)	56	24, 5.96	20, 5.60	Q96AX2	30

Additional Information of MALDI Identified Upregulated Proteins

Protein name	*Mascot Score >55*	*Theoretical Mr/pI (kDa/pH)*	*Experimental Mr/pI (kDa/pH)*	*Accession Number*	*Sequence coverage (%)*
Cleavage stimulation factor (CSTFT) 64 kDa subunit, tau variant (CSTFT)	60	64, 6.79	50, 5.60	Q9H0L4	12
Heterogeneous nuclear ribonucleoproteins C1/C2 (HNRPC)	59	33, 4.95	50, 5.60	P07910	20
26S protease regulatory subunit 7 (PSR7)	60	49, 5.71	50, 5.60	P35998	25
Brain finger protein BFP/ZNF179 - human JC7155 (RING finger protein 112, RN112)	64	69, 8.88	50, 6.20	Q9ULX5	21
Fascin (Singed-like protein) (55 kDa actin-bundling protein) (p55), FSCN1	74	54, 6.81	55, 5.20	Q16658	26
Complement C1q tumor necrosis factor-related protein 9 (CIQT9)	64	31, 7.88	40, 5.20	P0C862	29
Tryptophan/serine protease (YHOO4)	62	39, 7.95	38, 5.10	Q6UWB4	28
Pyruvate kinase (KPYM)	58	58, 7.96	40, 5.70	P14618	22
NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, assembly factor 1 (Complex intermediate associated protein 30) (CIA30)	57	37, 7.11	37, 7.10	Q9Y375	22
Mutant desmin (DESM)	56	53, 5.21	50, 5.90	Q549R9	18
Solute carrier family 25 member 16) (GDC)	58	36, 9.84	27, 5.90	P16260	35

Additional Information of LC-MS/MS Identified Upregulated Proteins

Protein name	*Mascot*	*Theoretical Mr/pI (kDa/pH)*	*Experimental Mr/pI (kDa/pH)*	*Accession No*	*Sequence coverage (%)*
Haptoglobin (HPT)	213	45, 6.13	58, 4.90	P00738	12
Chitotriosidase (CHIT1)	209	50, 6.29	48, 5.81	Q13231	10
Beta-2-glycoprotein 1 precursor (APOH)	171	40, 8.34	60, 6.00	P02749	10
Lysosomal Acid Phosphatase (PPAL)	55	48, 7.07	60, 6.00	P11117	18

Additional Information of LC-MS/MS Identified Downregulated Proteins

Protein name	*Mascot*	*Theoretical Mr/pI (kDa/pH)*	*Experimental Mr/pI (kDa/pH)*	*Accession No*	*Sequence coverage (%)*
Complement C3 precursor (CO3)	2909	188, 6.02	76, 6.30	P01024	24
Alpha 1 antitrypsin precursor (A1AT)	406	58, 4.79	60, 4.70	P01009	16
Serotransferrin (TRFE)	512	79, 6.81	40, 5.00	P02787	13
Fibrinogen (FIBB)	809	56, 8.54	36, 4.75	P02675	32
Zinc alpha 2-glycoprotein precursor (ZA2G)	73	34, 5.57	35, 5.40	P25311	15

Appendix E

Table of Combined Mass Spectrometry Data with Gel Spot Data

Functional category	Protein	Up or Down regulation	Fold Expression in Non-survivors	Expression Median		Confidence interval	
				Survivors	Non-survivors	Survivors	Non-survivors
Cellular defense proteins	Complement C3 precursor	↓	4.77	0.059	0.012	0.002 - 0.117	0.005 - 0.020
	Chitotriosidase	↑	5.14	0.032	0.130	0.000 - 0.066	0.047 - 0.213
	Complement C1q tumor necrosis factor-related protein 9	↑	3.42	0.023	0.083	0.009 - 0.038	0.030 - 0.135
Chaperones	T-complex protein 1 subunit zeta	↓	2.75	0.022	0.008	0.006 - 0.038	0.002 - 0.014
Metabolic enzymes	Phosphoglucomutase-like protein 5	↓	4.87	0.047	0.012	0.010 - 0.085	0.005 - 0.019
	Glyoxalase domain-containing protein 4	↓	3.57	0.052	0.013	0.000 - 0.113	0.007 - 0.018
	26S protease regulatory subunit 7	↑	2.46	0.009	0.027	0.004 - 0.014	0.000 - 0.053
	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9, mitochondrial	↑	2.76	0.007	0.022	0.002 - 0.013	0.000 - 0.044
Translation	Retinoic acid receptor RXR-gamma	↓	2.46	0.057	0.025	0.023 - 0.091	0.015 - 0.035
	Cleavage stimulation factor (CSTFT) 64 kDa subunit, tau variant	↑	3.30	0.035	0.118	0.005 - 0.066	0.038 - 0.197
	Heterogeneous nuclear ribonucleoproteins C1/C2	↑	2.46	0.026	0.068	0.011 - 0.042	0.030 - 0.106
	Zinc finger protein 179	↑	2.02	0.033	0.053	0.017 - 0.049	0.023 - 0.083
	Zinc finger protein 1	↓	2.02	0.179	0.075	0.094 - 0.264	0.028 - 0.122
	Eukaryotic translation initiation factor 2, subunit 2 beta	↓	2.18	0.051	0.026	0.025 - 0.077	0.009 - 0.044

Table continued.

Functional category	Protein	Up or Down regulation	Fold Expression in Non-survivors	Expression Median		Confidence interval	
				Survivors	Non-survivors	Survivors	Non-survivors
Transporters	Serotransferrin	↓	5.47	0.117	0.027	0.057 - 0.178	0.005 - 0.050
	Solute carrier family 25 (member 16)	↑	3.97	0.007	0.027	0.001 - 0.013	0.000 - 0.056
Glycoproteins	Beta-2-glycoprotein 1 precursor	↑	3.61	0.029	0.086	0.001 - 0.057	0.010 - 0.161
	Zinc alpha 2-glycoprotein precursor	↓	2.87	0.061	0.022	0.010 - 0.112	0.008 - 0.036
G proteins of the Ras family	Ras-related protein Rab-37	↓	3.82	0.014	0.005	0.001 - 0.027	0.000 - 0.010
Globins	Haptoglobin	↑	2.91	0.025	0.050	0.000 - 0.051	0.002 - 0.098
Kinases	Brain-enriched guanylate kinase-associated protein (BEGAIN)	↓	5.01	0.083	0.019	0.000 - 0.203	0.006 - 0.032
	Pyruvate kinase	↑	2.06	0.035	0.063	0.014 - 0.055	0.000 - 0.129
Proteases	Tryptophan/serine protease	↑	3.07	0.007	0.022	0.000 - 0.014	0.006 - 0.037
Phosphatases	Lysosomal Acid Phosphatase	↑	2.68	0.018	0.050	0.000 - 0.039	0.016 - 0.084
	Serine/Threonine phosphatase 2-alpha 65K regulatory chain	↓	2.68	0.104	0.045	0.071 - 0.138	0.004 - 0.086
	Serine/threonine/ tyrosine-interacting-like protein 1	↓	2.24	0.195	0.085	0.121 - 0.269	0.056 - 0.115
Membrane and skeletal proteins	Fibrinogen	↓	2.51	0.038	0.013	0.014 - 0.062	0.005 - 0.021
	Fascin	↑	3.02	0.039	0.095	0.022 - 0.056	0.029 - 0.162
	Mutant desmin	↑	2.71	0.023	0.053	0.016 - 0.030	0.022 - 0.083
	Ankyrin repeat domain-containing protein 42	↓	2.51	0.061	0.032	0.029 - 0.093	0.001 - 0.055

Table 1 contd.

Functional category	Protein	Up or Down regulation	Fold Expression in Non-survivors	Expression Median		Confidence interval	
				Survivors	Non-survivors	Survivors	Non-survivors
Others	Alpha 1 antitrypsin precursor	↓	2.97	0.021	0.010	0.005 - 0.037	0.000 - 0.024
	Nuclear localized factor 1	↓	2.09	0.097	0.055	0.000 - 0.210	0.000 - 0.118
	Human Serum Albumin	↓	2.01	0.392	0.169	0.130 - 0.654	0.000 - 0.346
Unknown	Cancer associated gene 1	↓	2.45	0.070	0.027	0.028 - 0.112	0.006 - 0.048

The table above shows the functional clustering of the proteins identified from mass spectrometry. The expression of each protein was based on the normalized volume of each of the protein spots when identified as a match in all the gels. This was then used to create an averaged gel which involved comparing the non-survivors to the survivor gels. The median of each normalized volume of the proteins from the samples is shown along with the 95% confidence intervals for the normalized volumes.